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EXPERIMENTS ON THE EFFECTS OF TEMPERATURE AND HUMIDITY ON THE SURVIVAL OF *HALOTYDEUS DESTRUCTOR* (Tucker), ACARINA fam. PENTHALEIDAE

by

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(Submitted for publication 28th October, 1936.)

I. INTRODUCTION.

It is well recognized that temperature and atmospheric humidity are important factors affecting the distribution of terrestrial animals and fluctuations in their numbers. Temperature is not only important as a direct factor, but also indirectly, since it influences atmospheric humidity. The thin cuticle of the Earth Mite (*H. destructor*) appears to be permeable to water, for when the animal is exposed to a dry atmosphere it loses moisture rapidly. Moisture is also lost through the two stigmata, the tracheal system being fairly extensive. *H. destructor* spends much of its time on the leaves of plants, where it is less affected by air humidity, because moisture lost by evaporation is replaced by the juices absorbed from the leaf tissues. While on the soil, the mite tends to remain in damp, sheltered situations where the humidity is high.

The aim of the experiments described here has been to ascertain the moisture requirements of the mite, at various temperatures, in the presence or absence of its food-plant. Most of the experiments were carried out with adult mites; in some instances nymphs were used for comparison with the adults.

The general method employed has been to expose a number of mites to known combinations of heat and moisture, and to observe the percentage dead at intervals, special attention being given to the time of exposure necessary to obtain fifty per cent. mortality. This time of exposure is referred to as the "survival period". A somewhat similar method was used by Davies (1928) with Collembola. The results obtained by Davidson (1934a) with *Smynthurus viridis* L. are of particular interest in the present instance since *S. viridis* inhabits much the same microenvironment as *H. destructor*. Numerous workers have mapped out lethal zones of temperature and humidity for various species of insects, without taking into account the length of exposure required to produce the lethal effect; many workers

have studied the effect of temperature on the velocity of development. Apparently very little work of this kind has been done with Acarina. The only experiments that the writer has been able to trace are those of Newstead and Morris (1920) on the lethal low humidity content of flour for the Tyroglyphid mite *Aleurobius farinae*.

Throughout the paper, the abbreviations "r.h." and "s.d." are used for the terms "relative humidity" and "saturation deficit". The importance of atmospheric humidity lies chiefly in its effect upon evaporation from the animal's body. Hence s.d. is a more useful index of humidity than is r.h., for the evaporating power of the air is almost proportional to its s.d., if wind velocity and atmospheric pressure are constant. Aspects of this question have been treated by Buxton (1931), Davidson (1934a, 1934b, 1934c), Mellanby (1935), Ramsay (1935a, 1935b) and others.

In the present series of experiments, s.d. has been used as the standard measure of humidity. The results show that the effects of desiccation on *Halotydeus destructor*, over the temperature range, 1° to 35° C., are correlated much more closely with s.d. than with r.h.

II. METHODS AND APPARATUS.

a. *Collection of Mites.*

The mites used in the experiments were collected about the grounds of the University of Western Australia, Crawley. The food-plants in these areas were clovers (*Trifolium procumbens*), capeweed (*Cryptostemma calendulaceum*) and milk thistle (*Sonchus oleraceus*). The mites were shaken direct from the food plant into a vessel of water; when this was not practicable they were sucked up from the soil surface with an aspirator and transferred to water. They were kept on the water for periods varying up to six hours (11 hours in one case). Tests showed that the effects of this treatment were unimportant and could be ignored in the subsequent experiments.

b. *Apparatus.* (Figure 1).

The apparatus used to produce a steady air-flow was a modification of the aerating apparatus described by Cannon and Grove (1927). The reservoir *R* was supplied by an inlet *T* from the tap and was provided with an overflow outlet *F*. The vertical glass tube *AM* received water from the reservoir through the supply tube *S*. This water was hindered in its fall by a twist in the tube at *L*, so that bubbles of air from the air-inlet *A* were carried down the tube to the jar *J*. This water and air falling into the jar caused the pressure to increase until air was forced out at *N* into the apparatus, while the water in the bottom of the jar was forced out at *O*, the height of which had to be adjusted according to the pressure required to force air through the apparatus. (In the later 1936 experiments this circulating apparatus was replaced by a Dinton electric air-pump, which was more satisfactory because it required no manipulation.) From *N* the air-stream passed through a capillary resistance *B* in a Venturi meter, which was calibrated so that the speed of air-flow could be read off as a proportion of the difference in level of the two water columns in the U-tube *D*.

The air next passed through the rubber-stoppered bubbler *W*, which in some of the experiments contained a saturated solution of an appropriate salt. In others, *W* contained water. The air-stream, having been conditioned to a known constant r.h. by bubbling through *W*, was passed through an experimental chamber *a* containing mites. The details of the structure of such a chamber are shown in figure 1a: a cylindrical glass tube of internal diameter 24 mm. was fitted

at each end with a rubber stopper *r*, through the centre of which passed a small glass tube *t*. A circle of silk *s* surrounded the inner end of each stopper and prevented the exit of the mites without presenting any serious hindrance to the air-flow. After emerging from this chamber, the air was passed through a second bubbler *E* which contained a saturated solution of an appropriate salt and had the effect of reducing the humidity of the air to a known constant value before it was passed over the mites in a second chamber *b*, similar in construction to *a*. Then the humidity of the air was still further reduced by passing through another saturated salt solution in the bubbler *F*, whence it traversed the chamber *c* and escaped through a valve *G* which contained liquid paraffin or olive oil. A pressure system such as this is preferable to one in which the air is sucked through the apparatus, because any small leaks will be outwards and therefore will not affect the humidity in the apparatus.

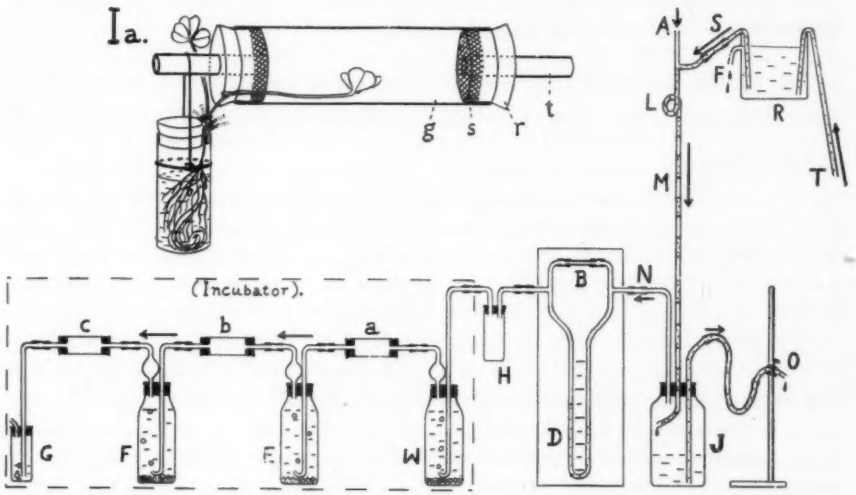

Fig. 1. Diagram of apparatus. 1a, Sketch of an experimental chamber on a larger scale, showing also the method of inserting clover leaf. For description see text.

Except in a few experiments at room temperature, the bubblers and experimental chambers were kept in a Hearson biological incubator. Care was taken to ensure that the solutions should be saturated and at the correct temperature before each experiment began. For checking the reliability of the bubblers, a dew-point apparatus was set up. The formation of dew was observed through a horizontal microscope. The bubblers that were tested in this way gave r.h. closely approaching those theoretically required, provided that there was an excess of crystals, and that care had been taken in the first place to make up a completely saturated solution. The following is the list of salt solutions employed. The r.h. quoted are those given by Buxton and Mellanby (1934) for 20°C.; those inserted in parenthesis are the results of tests with the dew-point apparatus at room temperature: Ca_2HPO_4, $2H_2O$, 95 p.c.; KCl, 86·5 p.c. (86 to 88 p.c.); NaCl*, 78·3 p.c. (77·5 to 80 p.c.); K_2CO_3, $2H_2O$*, 44 p.c. (44 to 45 p.c.); $MgCl_2$, $6H_2O$*, 34 p.c. (34 p.c.);

* In these three bubblers the air after emerging from the jet travelled up through the solution inside a wide glass spiral. This ensured better contact between the air and the solution.

CH_3COOK, 20 p.c. (23 to 24 p.c.); $ZnCl_2$, 10 p.c. When a r.h. approaching 0 p.c. was required the air-stream was passed through concentrated sulphuric acid.

It was desired, in some experiments, to supply the mites with a leaf of a living food-plant. This was inserted into the chamber as shown in Fig. 1a, the petiole being laid in a groove cut in the rubber stopper. The roots of the plant (*Trifolium subterraneum*) dipped into water in a small suspended vessel. The chamber containing the leaf was inserted between *c* and *G*, so that any leakage through the groove in the rubber stopper would be negligible. The group of mites in *c* served as controls.

e. *Procedure.*

The number of mites used in each experimental chamber was 20 to 100, but usually about 50. In most experiments four or five chambers were used; this is shown in the tables. Except in the more lengthy experiments observations on the mites were made at intervals of about one hour. In the incubator experiments opening the glass door resulted in a temporary drop in temperature amounting to 2° or 3°C., sometimes of several minutes duration.

III. MINOR FACTORS AFFECTING THE SURVIVAL PERIOD.

Consideration was given to the effect of additional factors in the experiments.

a. *Rate of air-flow.*

The rates of air-flow in the experiments ranged from 1·25 to 10·5 litres per hour. The cross-sectional area of each experimental chamber was 4·52 sq. cm. Hence the speed of the air (disregarding the irregular distribution in the chamber) was 277 cm. per hour at the lower limit, and 2,324 cm. per hour at the highest rate of flow.

Some experiments were carried out with a view to testing the effects of air-movement within these limits (Table 1). An air-stream of known velocity was passed through an experimental chamber containing mites; on emerging from this it was divided by a Y-tube and each of the two streams passed through a chamber also containing mites. In experiment 3, the two air-streams were the same; in experiments 1 and 2 they were in the ratio 2 : 1. The effect of the air-stream on the rate of evaporation and the survival period of the mites was negligible (Table 1).

TABLE 1.

Results of Experiments on the Effect of Rate of Air-flow on the Survival Period of Adult Mites.

Exp. No.	Exptl. Chamber.	No. of mites used.	Temp. °C.	Approximate Sat. Def. in mm. Hg.	Speed of air-flow in litres per hour.	Speed of air-flow through exptl. chamber in cm. per hour.	Survival Period: time in hours for 50% mortality.
1	a	47	26	14·5	7·5	1,650	7·5
	b	72	26	14·5	5	1,100	8·25
	c	57	26	14·5	2·5	550	9
2	a	39	30	17·5	10·5	2,310	6·75
	b	52	30	17·5	7	1,540	6·5
	c	58	30	17·5	3·5	770	6
3	a	95	15	7·4	5	1,100	14
	b	79	15	7·4	2·5	550	14

b. *History of the mites prior to the experiments.*

i. *1935 Experiments.* The mites used for the 1935 experiments were collected during August to October from a small area (about 100 yards by 5 yards). During this period the annual vegetation reached maturity and much of it began to wilt and die off. Accordingly, the mites became restricted to the more protected situations in the shade of trees, or under dense vegetation. Although the plants and also the soil dried out in many places, it is probable that the physical conditions in the local situations inhabited by the mites did not change very much; the rise in day temperature from the beginning to the end of the period was less than 5° C. The results obtained with mites collected near the beginning and towards the end of this period exhibited no marked discrepancies in survival periods.

The optimum humidity for the mites under well-ventilated conditions and in the absence of food is about 100 p.c. r.h. Experiments 4 to 8 indicate that prolonged exposure to a wet atmosphere without adequate ventilation produces harmful effects inasmuch as the mites afterwards show a lowered resistance to desiccation. Mites collected in the field were subjected to the following treatment.

Experiment 4: A 6-inch flower-pot containing growing subterranean clover plants was kept in a box sunken in the ground and shaded. The flower-pot was covered with muslin supported on a wire frame 8 inches high. A number of mites was kept under this frame for one week, the soil being well watered. Then (22/10/35) 46 of these mites were put into an experimental chamber; a control chamber was supplied with 67 mites collected under clover in the field. Both chambers were kept at 30° C., and supplied with air at a s.d. of 4·5 mm. (85 p.c. r.h.).

Result: Survival period of mites from flower-pot, 6·25 hours; controls, 11 hours.

Experiment 5: Mites were kept for several weeks under conditions as in experiment 4, except that they were covered by a hurricane lamp-glass with muslin top instead of by a wire frame. On 22/10/35, 24 of these mites were put into an experimental chamber; 75 mites collected under clover in the field were placed in a control chamber. Both chambers were kept at 30° C., and supplied with air at a s.d. of 20 mm. (r.h. 34 p.c.).

Result: Survival period of mites from flower-pot, 2·5 hours; controls, 5·5 hours.

Experiment 6: The mites were kept for 48 hours in the laboratory at room temperature in a closed Petri dish containing water of which the surface was almost covered with floating clover leaves. They were then placed in air of different humidities at room temperature (mean 15° C.). The control experiment was made three days earlier with a set of mites collected in the same situation but 25 hours earlier. Three chambers at different humidities were employed.

Results: (a) s.d. 9·6 to 10 (r.h. 24 p.c.). 27 mites from Petri dish: survival period 9·5 hours; 19 control mites, 11 hours. (b) s.d. 8·5 to 8·6 (r.h. 34 p.c.).

47 mites from Petri dish: survival period, 11·5 hours; 21 control mites, 14·5 hours. (c) s.d. 7 to 7·5 (r.h. 45 p.c.). 42 mites from Petri dish: survival period, 12·5 hours; 20 control mites, 15 hours.

Experiment 7: The mites were kept for 48 hours in the laboratory on subterranean clover plants growing in water, the whole being enclosed by a glass jar. 38 of the mites were then subjected to an air-stream having a s.d. of 3·25 mm. (r.h. 78 p.c.), at room temperature (mean 17·5° C.). Another experiment was made six days later, when 99 mites were collected, kept six hours on water, and subjected to a similar air-stream at mean room temperature 16·5° C.

Result: Survival period of mites from first experiment, 63 hours; second experiment (controls), 93 hours.

Experiment 8: Sixty mites collected on 24/10/35 from damp sand with short clovers, in shaded position; the soil was particularly moist owing to drainage from recent rains. 63 control mites were collected from slightly damp sand under clovers in unshaded situation. Both lots were kept at 32° C. and subjected to an air-stream of s.d. 19 mm. (45 p.c. r.h.).

Result: Survival period for mites from moist environment, 4·5 hours; controls, 5·5 hours.

Experiment 8 represents an extreme case, but it shows that even in the small area where the experimental mites were collected, diversity in the micro-environments may result in the occurrence of mites of different powers of resistance to desiccation. It would be advisable to adopt some method of standardizing the mites to be used in the experiments; but it was found impossible to maintain suitable conditions of moisture, food-supply and ventilation, while at the same time preventing the mites from wandering away.

ii. *1936 Experiments.* Experiments were carried out during the period June to early August, 1936. It was found impracticable to collect mites from the plot of ground used the previous year. The area selected was equally sandy, but flat and low-lying. The chief food-plant was milk-thistle and, to a lesser extent, capeweed. There was a general tendency for the survival period in the 1936 experiments to be shorter than in 1935. This tendency is seen in Figs. 5, 7 and 9. It seems reasonable to suggest that this difference was due to the weather; the mites used in 1936 had developed earlier in the winter, under wetter conditions and at a lower temperature than those used in 1935; they were probably less exposed to desiccation in the field.

IV. RESULTS OF EXPERIMENTS WITH MITES IN ABSENCE OF FOOD PLANT.

i. *With temperature range 10° C. to 35° C.*

Fig. 2 illustrates records of four experiments in which atmospheric saturation deficit was roughly constant, while temperature was different in each experiment.

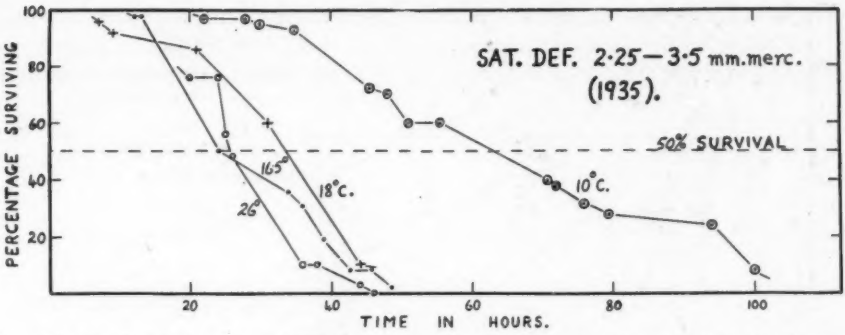

Fig. 2. Survival curves at different temperatures, when saturation deficit is about constant. Each curve shows the dying off of a group of about 50 mites in one experimental chamber.

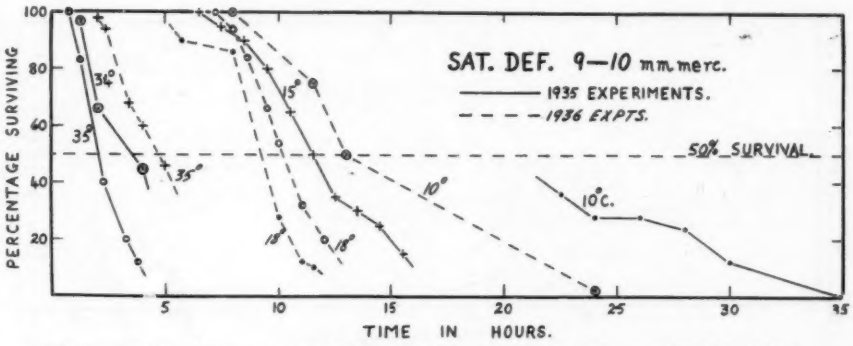

Fig. 3. Data analogous to those in Fig. 2, but at a different constant saturation deficit.

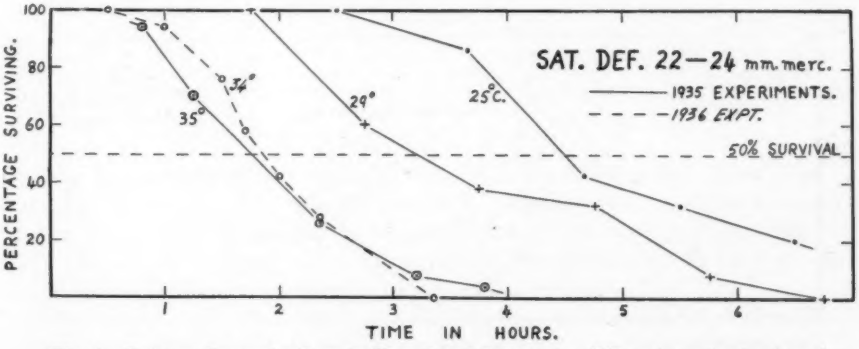

Fig. 4. Data analogous to those in Figs. 2 and 3, but at a different constant saturation deficit.

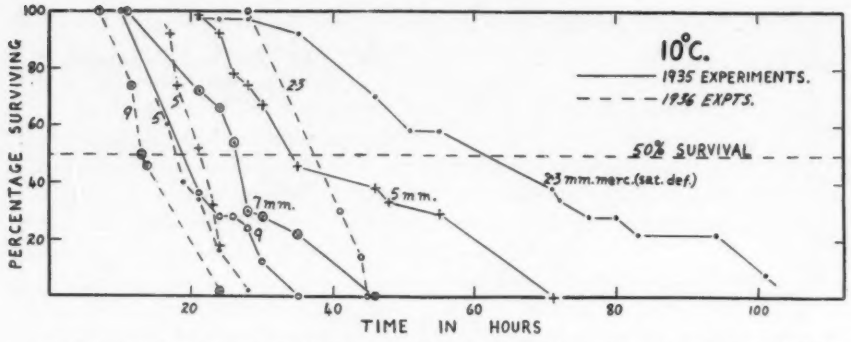

Fig. 5. Survival curves at different humidities, when temperature is constant (10°C.). Each curve shows the dying off of a group of about 50 mites in one experimental chamber.

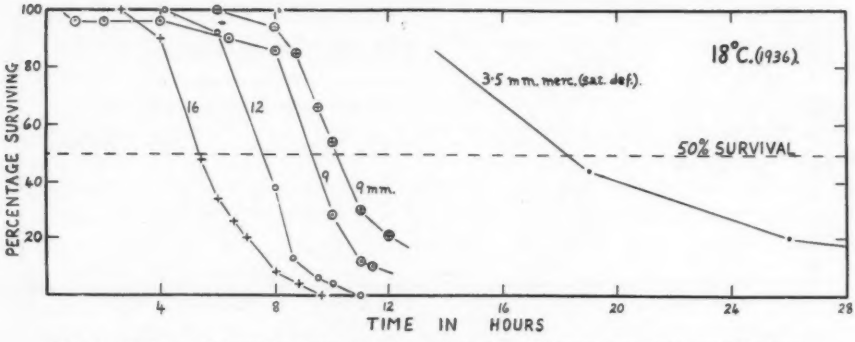

Fig. 6. Data analogous to those in Fig. 5, but at a different constant temperature.

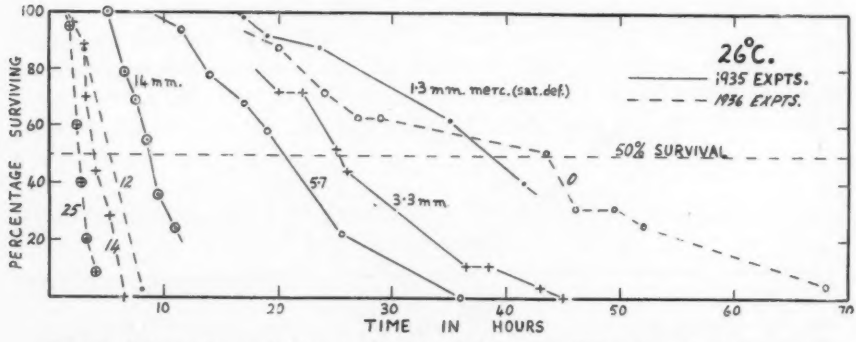

Fig. 7. Data analogous to those in Figs. 5 and 6, but at a different constant temperature.

Figs. 3 and 4 represent the temperature effects at two other humidities. Fig. 2 shows that there is no appreciable change in temperature effect from 16·5° C. to 26° C.; with this exception, the higher the temperature, the shorter is the survival period.

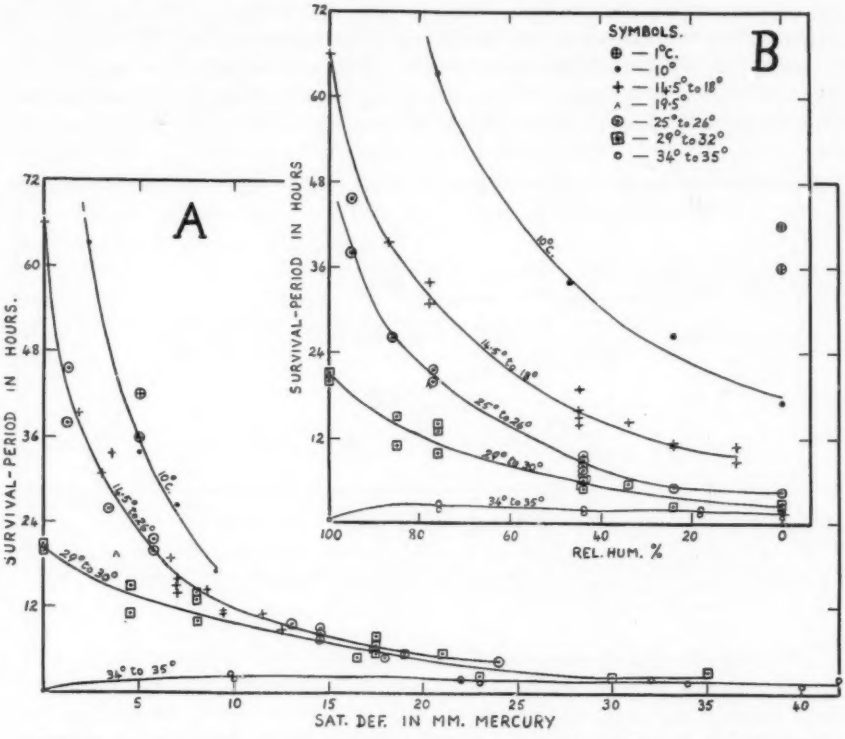

Fig. 8. A. Curves for survival period at different saturation deficits and at the various temperatures indicated by the symbols. 1935 data only. B. The same data plotted against relative humidity instead of saturation deficit. Each point represents the survival period (50% mortality) of a group of mites, usually about 50 in number.

Fig. 5 shows records of experiments at 10° C. with different humidities. Figs. 6 and 7 illustrate similar data for 18° and 26° C. The three figures illustrate that, the lower the saturation deficit, the longer is the survival period. Figs. 5 and 7 also illustrate the tendency of the mites in the 1936 experiments to succumb to desiccation earlier than those used in the 1935 experiments.

The results of the 1935 experiments, including many carried out at temperatures and humidities other than those covered by Figs. 2 to 7 are summarized in Fig. 8A. Each point in this diagram indicates the survival period of a group of

mites, usually about 50 in number, at the temperature and s.d. values indicated. As shown by the curves, the data fall into four temperature groups. The most interesting feature is that the data for temperatures from 14·5° to 26° C. are grouped together (compare Fig. 2); temperature differences within these limits seem to have practically no effect. The points for temperatures of 29° and 30° also fall near this group, showing that the effect of a rise in temperature from 26° to 30° is small. The effects of humidity are clearly shown: the higher the humidity (i.e. the lower the s.d.) the longer is the survival period, and the mites survive longest when the humidity is at or near to saturation point.

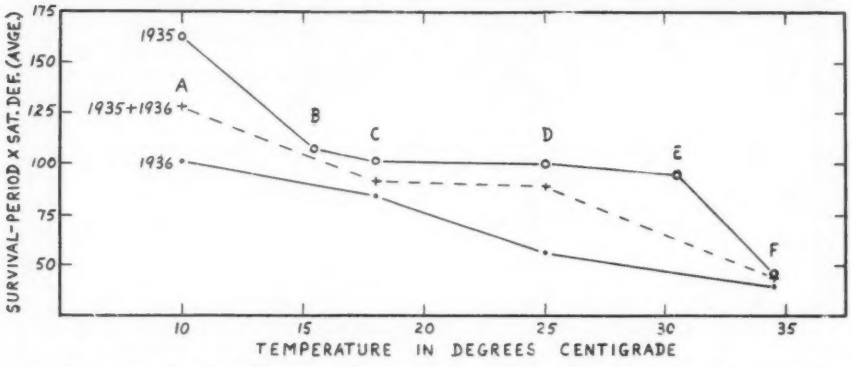

Fig. 9. Illustration of the effect of temperature on the survival period. Average values of the product, survival period × saturation deficit, at different temperatures: A, 10°C.; B, 14·5° to 16·5°; C, 17° to 19·5°; D, 24° to 26°; E, 29° to 32°; F, 34° to 35°.

Fig. 8B contains the same data as Fig. 8A, expressed in terms of r.h. It is evident from Fig. 8B that for any one r.h. value, the length of the survival period varies considerably with temperature; with saturation deficit (excluding low values), the variation in survival period is much smaller. Over the temperature range 10° to 35° C., the correlation between survival period and s.d. is better than the correlation between survival period and r.h. This is particularly noticeable over the range 14·5° to 26° C. If the survival period were dependent only on the rate of evaporation and not on temperature, one would expect the value *survival period* × *s.d.* to be fairly constant throughout the temperature range; but since the survival period is also affected by the temperature, this product is greatest at the lowest temperatures, and least at the highest temperatures. The lowering of body temperatures at low humidities as a result of evaporation may be ignored.

The relationship between temperature and the value of the product, *survival period* × *s.d.*, is shown clearly in Fig. 9, in which average values for this product are plotted for a number of temperature zones into which the complete temperature

range has been broken. The curve for the 1935 experiments is the most significant of the three, since it is based on the most complete series of data. It shows that, between the limits 15° and 30° C., the survival period is affected very little by temperature changes, and not at all between 18° and 26°. Within these limits the survival period tends to be inversely proportional to the dryness of the air, as measured by s.d. At 15° the survival period is slightly longer than at 18° (a point that was not brought out by Figs. 2 to 8), while a drop in temperature from 15° to 10° results in a marked increase of survival period. At 30° the survival period is a little shorter than at 26°, while a rise to 34° causes a marked shortening of the survival period. The curve for the 1936 experiments shows that the average survival period was shorter at all temperatures from 10° to 34°. The form of the curve differs from that of the 1935 curve chiefly because of the relatively short survival periods recorded in the few experiments at 25°.

A few experiments were conducted with nymphs; the results of these are set out in Table 2 for comparison with the results for adults (Figs. 8A, 8B). They show a comparatively low resistance to desiccation.

TABLE 2.

Survival Period for Nymphal Mites.

Exp. No.	Exptl. chamber.	No. of mites used.	Instar †	Temperature °C.	Rel. Hum. %	Sat. Def. in mm. mercury.	Survival Period (50 p.c. mortality) in hours.	Survival Period for adults under same conditions.	Survival Period × Sat. Def.
37	a	6	2 and 3	10	100	0	96+	—	—
	b	4	,,	10	76	2·25	28	(63)	63
	c	3+	,,	10	10	7	10+	(26·5)	70+
	d	3+	,,	10	0	9	10+	(17)	90+
38	a	12	,,	18*	100	0	60+	—	—
	b	10	,,	18*	78	3·3	11	(36)	37
	c	10	,,	18*	44	8·5	7	—	59
39	a	10	,,	19·5*	44	9·5	3	—	28
	b	23	,,	19·5*	24	12·5	3	—	37
	c	?	,,	19·5*	0	17	3	—	51
40	a	23	4	26	77	5·75	16	(20) (21·5)	92
41	a	16	3	26	44	14·5	3·5	(7·5) (9)	51

Temperatures marked * are mean values of fluctuating room temperatures.

† It has been assumed that there are five instars in the complete life history. This is very probably correct, but has not yet been conclusively proved.

ii. *Lethal Effect of High Temperatures.*

In order to test the effects of high temperatures, mites were placed in small corked vials in a saturated atmosphere. The vials were immersed in water baths

at various temperatures. The survival periods at various lethal temperatures are given in Table 3. The period of exposure at a high temperature necessary to cause death is considerably shorter than the "*survival period*" obtained with mites kept indefinitely at that temperature.

TABLE 3.

Survival Periods at Various Lethal High Temperatures in Presence of Excess Moisture.

No. of mites used.	Stage in life history.*	Temperature °C.	Approximate average time for death in minutes (50 p.c. mortality).	Time in minutes for death of all mites.
?	Adults and nymphs in all instars	29	120—	1020+ (unfinished)
30	Adults	35	2·5	5
95	Adults and nymphs in all instars	35	—	12+
10	Nymphs, 4th instar	38	0·5—	1·5
20	Adults	38	1	2·5
100	Adults and nymphs in all instars	40	2	2·5
25	Adults and nymphs in all instars	50	0·3	0·3

* See note to Table 2.

For example, the table shows that some of the adult mites survived at 38° C. as long as 2½ minutes. But when mites were exposed to this temperature for only half a minute and removed again to cooler surroundings (17° C.) they nevertheless died within a few minutes.

iii. *Survival Period at Low Temperatures.*

Some mites were enclosed in small vials in a saturated atmosphere; the vials were kept in an ice-box, the temperature in the vials being 1° C. Under these conditions the survival period for adults and nymphs of the second and third instars was found to be more than 6 days.

Similar vials were half-filled with dry calcium chloride, which was covered by a loose pad of cotton wool. Mites were put into the vials, above the cotton wool, and subjected to a temperature of 1° C. At this temperature and humidity (about 0 p.c. r.h.) the survival period for adults was found to be 36 hours in one experiment, 42 hours in another. At 10° C. and a similar s.d. (5 mm.), the survival period was 34 hours, so that the mites appear to be more resistant to desiccation at 1° C. than at 10° C.

V. RESULTS OF EXPERIMENTS WITH MITES WITH FOOD-PLANT ACCESSIBLE.

When a food-plant was available (Fig. 1a), a proportion of the mites came to rest on it, and some at least of these fed upon it. With the rate of air-flow main-

tained in the experiments (see p. 4) it may be assumed that the presence of a single clover leaf in the chamber did not significantly affect the air humidity, except close to the leaf surface. A mite feeding on the leaf would be taking in moisture and would also experience a relatively moist atmosphere externally.

The proportion of mites on the plant was generally lower the higher the temperature (see Table 4); also, it was lower in the earlier stages than in the later stages of an experiment, when some of the mites had died and were therefore not included in the count. The estimated percentages in the third column of Table 4 are based on counts made at intervals during the experiments. The differences are not entirely due to temperature differences, since the s.d. is greater at the higher temperatures, and the mites are always restless in dry conditions.

TABLE 4.

Survival Period of Adult Mites when Food-plant is Accessible.

No. of mites used.	Estimated average % of living mites on plant during expt.	Temp. °C.	Rel. Hum. %.	Approx. Sat. Def.	Survival Period in hours (Time for 50% mortality). With plant.	Controls without plant.	At same temp. but sat. def.=0.
25	50%	10	47	5	168	18	160+
25	60%	10	0	9	78	13	160+
27	90%	15*	10	11·5	70	(10)	140+
24	60%	18	44	9	32	9·5	54+
25	60%	18	0	16	24	5·5	54+
25	40%	24	44	12·5	49	4·5	35 45·5
25	20%	26	0	25	4	2·5	35 45·5
30	20%	34	44	22	2·6	1·9	2·75
25	5%	34	0	40	1·6	1	2·75

* Mean of a fluctuating room-temperature.

These considerations offer a partial explanation of the observations shown in Table 4, that the effect of the presence of the plant in lengthening the survival period was greater, the lower the temperature. For example, the presence of the plant increased the survival period by 6 to 9 times at 10° C., 3 to 4 times at 18°, and 1·5 times at 34° C. It is evident that the presence of the food-plant does not greatly delay the lethal effect of high temperatures (34°), even when the effect is a relatively slow one of an hour or more. At such temperatures the mites are too restless to remain on the plant.

It may be inferred from Table 4 that the mites are killed more readily by loss of water by evaporation than by starvation.

VI. CONCLUSIONS AND DISCUSSION.

The results of the foregoing experiments are affected to some extent by the physical conditions experienced by the mites prior to the experiments. This is shown in the experiments described in section IIIb. It seems to be the only reasonable explanation of the greater susceptibility to desiccation shown by the mites used in the 1936 experiments, compared with those of 1935, and of the erratic results which were obtained from time to time (excluding the possibility of variations in genetic constitution).

Over the temperature range 10° to 35° C. the survival period is correlated more closely with s.d. than it is with r.h. This is to be expected, since s.d. is the major factor affecting evaporation. However, it is not to be expected that any simple relationship exists between s.d. and the rate of evaporation from the mites; the latter is complex and doubtless takes place through the integument of the animals as well as from the tracheae.

Comparing survival periods at different temperatures and similar values for s.d., it is found that, the higher the temperature, the shorter is the survival period, except between 18° and 26° C., over which range the temperature effect is almost constant. This applies from the lowest temperature tested (1° C.) up to 50° C., at which temperature the mites die in a fraction of a minute. Above about 30° to 32° C. it appears that the temperature effect becomes dominant and masks the effect of humidity. Fig. 8 shows that, at 34° to 35° C., the survival period is almost independent of humidity. It is possible that, at high saturation deficits, the relatively rapid evaporation lowers the temperature of the mite, so that loss of moisture by evaporation is offset by a reduction in temperature. This reduction in temperature would result in a lowering of the vapour pressure at the body surface, and so decrease the rate of evaporation until a balance were struck between the two opposing tendencies. At 34° C., *Halotydeus* is killed within a few hours even at optimum humidity (i.e. near saturation).

By comparing survival periods at different humidities but similar temperature, it is found that, the higher the humidity, the longer is the survival period; the length of the survival period tends to vary inversely as the s.d.; it is longest when the air is at or near saturation point.

It is interesting to compare these temperature and humidity requirements with those of certain Collembola which, like *Halotydeus*, live in moist situations under vegetation. Their tracheal systems are restricted or absent, and it appears that evaporation occurs through the integument. Davies (1928) studied the effects of various humidities, in the absence of food, on five species of Collembola. All of them, including *Smynthurus viridis* L. (commonly found together with *Halotydeus* in Australia), survived longer at higher humidities, and longest with the humidity at saturation point. Similar results with *Smynthurus* were obtained by Mac-

Lagan (1932). The published data for *Smynthurus* show that, at 25°, 30° and 35° C., it is more resistant to desiccation than *Halotydeus*; this explains why it usually persists longer in the field at the onset of summer. Davidson (1934a) states that for *Smynthurus* nymphs "at temperatures above about 25° C., harmful effects set in and temperature becomes the dominating or limiting factor. With *Halotydeus*, these effects set in above about 30° to 32° C. If, as seems likely in *Halotydeus*, evaporation occurs through the integument, then the greater susceptibility of the nymphs must be at least partly due to the fact that they are smaller than the adults, and therefore have a greater evaporating surface area in proportion to their volume; also the rate of evaporation per unit of area is greater for small surfaces than for larger ones.

Mites in air of low humidity survive longer when a food-plant is accessible, but not as long as mites kept without food in air that is almost or completely saturated; in other words, they are killed more readily by desiccation than by starvation. The effect of the food-plant in increasing the survival period is less at higher, and greater at lower temperatures; this appears to be chiefly if not wholly due to the fact that the mites are more restless at higher temperatures and greater s.d., with the result that fewer of them remain on the plant.

The above information should be useful in interpreting fluctuations in the Earth Mite population in the field. But it will be necessary to obtain definite data as to temperature and moisture in local, natural situations under various meteorological conditions and at different times of the day.

SUMMARY.

Experiments are described in which *H. destructor* was exposed in atmospheres of various combinations of temperature and humidity. The results obtained illustrate the influence of these factors on the survival rate; for this purpose the "survival period" is defined as the "time exposure" necessary to obtain 50 p.c. mortality with a group of about 50 mites in each experiment.

With atmospheres, having the same temperatures but different humidities, the survival period is longer the higher the humidity; the optimum humidity is near saturation. With atmospheres having the same saturation deficit but different temperatures, the survival period decreases with rise in temperature, except within the range 18° to 26° C., within which no difference in temperature effect was evident. Above 30° to 32° C. harmful effects of temperature occur, and become the dominating factor influencing the "survival rate"; at 34° C. the mites die within a few hours even when humidity is about the optimum; at 50° C. they die in a fraction of a minute.

These results have an important bearing on the irregular fluctuations in the numbers of *H. destructor* in nature.

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REFERENCES.

Beattie, M. V. (1928): Bull. Ent. Res., Vol. 18, part iv, p. 397.
Buxton, P. A. (1931): Bull. Ent. Res., Vol 22, part iii, p. 431.
Buxton, P. A. and Mellanby, K. (1934): Bull. Ent. Res., Vol. 25, part ii, p. 171.
Cannon, H. G. and Grove, A. J. (1927): J. Roy. Micr. Soc., Dec., 1927, p. 319.
Chapman, R. N. (1931): "Animal Ecology". McGraw-Hill Co., New York and London.
Davidson, J. (1934a): Coun. Sci. Ind. Res. Bull. No. 79.
Davidson, J. (1934b): Trans. Roy. Soc., Sth. Aust., Vol. 58, p. 33.
Davidson, J. (1934c): Trans. Roy. Soc., Sth. Aust., Vol. 58, p. 197.
Davies, W. M. (1928): J. Exp. Biol., Vol. 6, pp. 79-86.
MacLagan, D. S. (1932): Bull. Ent. Res., Vol. 23, p. 101.
Mellanby, K. (1935): Biol. Rev., Vol. 10, part iii.
Newstead, R. and Morris, H. M. (1920): Roy. Soc., London, Grain Pests (War) Comm. Rept. No. 8, 15 pp. (Summary in Rev. App. Ent., Vol. 9 Ser. A, part i, p. 11, 1921).
Ramsay, J. A. (1935a): J. Exp. Biol., Vol. 12, pp. 355-371.
Ramsay, J. A. (1935b): J. Exp. Biol., Vol. 12, pp. 373-383.
Swan, D. C. (1934): J. Agr. Sth. Aust., Vol. 38, pp. 353-367.
Uvarov, B. P. (1931): Trans. Ent. Soc., London, Vol. 79, part i, p. 85.

STUDIES ON THE OPOSSUM *(TRICHOSURUS VULPECULA)*

I. BLOOD ANALYSES AND LIPOID GLANDULAR CONSTITUENTS IN NORMAL AND LACTATING OPOSSUMS.

by

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Little is known functionally of the Australian marsupials, yet it might be considered that chemical investigations, such as have occupied the attention of many workers with common laboratory animals, might well be undertaken on these peculiar mammals, and that the comparative studies afforded might throw an interesting light on problems that now present many contradictory features.

Since the Australian opossum represents a special type of primitive mammal, it might be expected to exhibit significant differences in its chemistry which would be helpful in defining the underlying action of organs whose functions are obscured by more evolved or less typical chemical inter-relationships in other mammalian species. An illustration of these expected functional type differences is afforded by the work of Britton and Silvette (1935) on adrenal insufficiency. They showed that in the American opossum (*Didelphys virginiana*) and in the marmot (*Arctomys monax*), in contrast to common laboratory animals, the blood sodium chloride level increases, instead of decreases, after adrenalectomy, and the carbohydrate disturbances assume greater importance than in, say, the dog; hence the reactions of these lower mammals afford valuable evidence for the theory of adrenal control of carbohydrate metabolism.

The following studies were undertaken to initiate a general enquiry into the chemistry of the Australian opossum. Attention has been concentrated on the lipoids with a view to establishing normals for work along certain operative lines of investigation which will be described in a subsequent paper; analyses have been extended to cover such glands as are commonly regarded as storehouses or as factories of the lipoids, and estimations of the sugar, non-protein nitrogen and total phosphorus of plasma have been included for comparison with other mammals.

EXPERIMENTAL.

The animals were all kept in a single colony in a darkened room, and fed on a diet of eucalyptus leaves supplemented with a mixture of equal parts of bran, pollard, wheat and bone meal. All experiments were performed after a sixteen hours fast. The number of animals in each experimental group is necessarily very small, owing to the difficulty of obtaining and keeping a large number of these animals in comparable experimental conditions.

I had found, from comparative analyses, that the glands lost from 10 to 20 p.c. of their lipoid content when animals were kept under ether for 15 minutes. Hence, to avoid the lipoid solvent effect of anaesthetics and the consequent shifts of constituents due to altered cell and membrane permeability, animals were killed by drowning, and the blood and tissues taken immediately for analysis.

The blood was examined for its non-protein nitrogen, sugar, total phosphorus, total lipoid, total cholesterol, total fatty acid, and lipoid phosphorus contents. The blood was citrated and centrifuged immediately on withdrawal and the cells and plasma examined separately.

The tissues taken were liver, spleen, kidney and adrenals. In the case of the first three, representative samples of the entire gland were used; the adrenals were analysed whole, either separately or mixed. The exsanguinated glands were dried at 50° C. for three hours, then allowed to stand in a vacuum desiccator for 24–48 hours, till they had attained constant weight. They were then powdered and the lipoids extracted.

Methods.

For sugar, the colorimetric ferricyanide method of Folin as given by Peters and Van Slyke (1932) was used. The results are recorded inclusively as sugar, although the nature of the reducing substances in the blood of these mammals has not been determined.

Non-protein-nitrogen: A micro-Kjeldahl procedure, with subsequent nesslerization, was used.

Phosphorus: All *P* estimations were carried out in principle according to Bodansky's (1932) modification of the Deniges reaction. The preliminary conversion of the *P* to ortho-phosphoric acid gave a great deal of trouble. A mixture of H_2SO_4 and HNO_3, as in Neumann's (1902) wet-ashing method, is inapplicable here, as the presence of HNO_3 prevents the colour development. Addition of sodium sulphite to remove traces of HNO_3 was abandoned, as all samples of sodium sulphite tried gave a blue blank. $KMnO_4$ added drop by drop and redigested between each drop to avoid excess and precipitation of MnO_2, was found to be satisfactory, but too tedious to be useful. Kuttner and Lichenstein's (1932) procedure of adding H_2O_2 drop by drop was found to be of no value in tissue estimations, as larger quantities than three drops of H_2O_2 were found to give a blue blank. Finally potassium persulphate was found to be a satisfactory oxidizing agent and to permit quantitative colour development under the usual conditions, provided that excess acid is avoided.

$$K_2S_2O_8 + H_2O \rightarrow K_2SO_4 + H_2SO_4 + O.$$

If 0·3257 gm. $K_2S_2O_8$ (equivalent to 0·25 c.c. of 10N. H_2SO_4) and 0·75 c.c. of 10N. H_2SO_4 are added to the evaporated lipoid extract (or to the diluted blood), digestion can be carried out easily and the colour subsequently developed with stannous chloride and molybdic acid in the usual way.

Lipoid estimations: The lipoids were extracted from the dried gland, or from wet blood

samples by Bloor's 3: 1 alcohol: ether mixture. Total lipoids were determined gravimetrically; total "cholesterol" by the Liebermann-Burchard reaction; fatty acids according to Stoddart and Drury's method as given by Peters and Van Slyke, and lipoid phosphorus as outlined above.

NORMAL ANIMALS.

In this group, eight animals, four of each sex, were investigated: the range covered half-grown and adult animals of un-determined age.

TABLE 1A.

Blood Constituents of Normal Opossums.

Animal.	Sugar. mg./100 c.c.		N.P.N. mg./100 c.c.		Total P. mg./100 c.c.		Total Lipoids. mg./100 c.c.		Total Cholesterol. mg./100 c.c.		Fatty Acids. mmoles/ 100 c.c.		Lipoid P. mg./100 c.c.	
	Cells	Plasma	Cells	Plasma	Cells	Plasma	Cells	Plasma	Cells	Plasma	Cells	Plasma	Cells	Plasma
2 half-grown	76	118	36	16	70	23	1,400	1,600	155	148	1·05	1·22	9·6	10·2
males	83	123	45	16	110	32	1,580	1,700	160	157	1·10	1·29	13·4	13·3
2 adult males	75	126	60	33	96	17	1,560	1,700	170	165	1·10	1·63	14·0	12·0
	79	130	93	45	84	15	1,750	1,850	200	198	1·20	1·33	10·5	10·2
2 half-grown	85	128	56	18	85	21	1,660	1,800	180	184	1·49	1·70	11·4	11·0
females	83	133	76	32	89	16	1,490	1,750	173	177	0·99	1·20	12·8	10·9
2 adult females	87	110	72	37	76	22	1,590	1,640	179	175	0·98	1·25	18·0	16·0
	82	115	84	44	80	20	1,520	1,790	180	178	1·42	1·53	14·0	13·9

Sugar: There is about 60 p.c. as much reducing substance per unit volume in cells as in plasma. In other mammals, the cell: plasma ratios are: in swine and rabbits, about 1: 4; in cattle, about 1: 2; in guinea-pigs about 1: 1 (Shope, 1928).

Non-protein Nitrogen: The cells contain more than double the amount of N.P.N. in the plasma—the constituents making up this excess were not determined. The values are high, and tend to increase with age. It is of interest that Mitchell (1931) found high blood N.P.N. values in another Australian mammal—the *Echidna Aculeata.*

Total P.: The amount and distribution of total phosphorus corresponds to that in human blood.

Lipoids: The total lipoids are higher than the values (about 370–570 mg./100 c.c. plasma) given for lipoids in human blood by Bloor (1916) and Boyd (1933). This may be due to the different techniques employed. With the method used here, three samples of human plasma gave an average value of 1,250 mg./100 c.c. Page, Kirk *et alia* (1935) report that there is no change in the lipoid fractions in the blood of normal men with advancing years; Parhon, Ornstein and Sibi (1933) find that, in humans, all lipoid fractions increase with age. In the opossum no age nor sex differences are apparent above. The cholesterol distribution is fairly even between cells and plasma; the fatty acid is rather variable, ranging from 270 to 470 mg./100 c.c. (if one takes the average molecular weight of the fatty acids to be

277, as in human blood), and the plasma content is higher than that of the cells, while the lipoid P tends to be higher in the cells than in the plasma.

TABLE 1B.

Lipoid Constituents of the Glands of Normal Opossums.

Animal.	Gland.	Dry Weight. gm.	Total Lipoids. gm./100 gm.	Cholesterol. mg./100 gm.	Fatty Acids. mmoles/100 gm.	Lipoid P. mg./100 gm.
Males	Kidney		16·6	1,066	34·0	237
			12·3	1,116	23·3	248
			15·0	980	25·4	270
Females	Kidney		13·6	1,040	22·9	300
			14·2	1,120	27·2	290
			15·4	1 260	19·8	224
Males	Spleen		13·9	950	26·9	233
			14·7	1,070	24·8	216
			13·1	1,456	17·4	202
			12·2	1,308	16·8	260
Females	Spleen		14·5	1 390	20·7	240
			13·4	1,400	19·4	306
			14·2	1,404	17·2	258
			12·9	1,356	18·6	233
Males	Liver		13·6	1,385	16·9	229
			13·1	1,418	19·9	284
			20·2	827	25·4	219
			16·4	851	24·8	276
Females	Liver		15·8	924	25·6	334
			12·9	830	24·9	253
			13·6	843	24·3	248
			18·4	826	25·9	292
Males	Adrenals	R 0·0120	17·2	829	24·8	302
		L 0·0302	19·8	848	24·2	279
		R 0·0243	37·0	4,017	141	—
		L 0·0258	58·6	3,028	109	—
		R 0·0200	35·2	5,032	117	—
		L 0·0214	41·6	3,596	184	—
		R 0·0199	38·9	3,702	126	—
Females	Adrenals	L 0·0226	42·3	3,892	177	—
		R 0·0209	40·8	4,004	148	—
		L 0·0199	39·8	3,912	153	—
		R 0·0197	43·2	3,509	169	—
		L 0·0210				
		R 0·0190				
		L 0·0236				
		R 0·0200				

Gland Lipoids: It will be noted that the liver has a lower content of cholesterol per gram than the other tissues investigated and that no sex differences are apparent. Okey, Gillum and Yokela (1934) find, in other species, that there are differences between the liver lipoids of males and females which may be related to sex metabolism. The adrenals, as is usual, have a much higher relative content of total lipoid, cholesterol and fatty acid than the other glands. Their lipoid P

content was not included in the tables, as it was found to be erratically variable (400–1,000 mg.) over the whole range of experiments conducted: no apparent correlation was possible with the fatty acid or cholesterol, or with any set of circumstances; hence no conclusions can be drawn. Since the phospholipoids play an active rôle in fat metabolism, and since the adrenals are credited with a mechanism affecting the phosphorylation of lipoids, their variable phospholipoid content may be a reflection of this activity: comprehensive data would be needed to establish this. The right adrenal, which is usually aberrant and adherent to the liver, is smaller than the left, though in the one case where separate analyses were made, its cholesterol and fatty acid content per gram were rather higher; thus its total cholesterol and fatty acid content approximates that of the left. No age nor sex variations of the adrenals were noticeable in this range of animals—in other species it has been shown that the adrenals exhibit certain sex variations.

It is of interest to compare the above findings with those in lactating animals.

LACTATING ANIMALS.

In this group three females were investigated. A had an embryo about 1 inch long on the teat in the pouch, B and C had young three to four months old on their backs. On post-mortem, A and B were found to be healthy, although very fat; C was grossly fat, and had a yellow, fatty liver containing spongy infarcts.

TABLE 2A.

Blood Constituents of Lactating Opossums.

Animal.	Sugar. mg./100 c.c.		N.P.N. mg./100 c.c.		Total P. mg./100 c.c.		Total Lipoids. mg./100 c.c.		Cholesterol. mg./100 c.c.		Fatty Acids. mmoles/ 100 c.c.		Lipoid P. mg./100 c.c.	
	Cells	Plasma	Cells	Plasma	Cells	Plasma	Cells	Plasma	Cells	Plasma	Cells	Plasma	Cells	Plasma
A	136	230	84	33	84	17	1,690	1,710	172	179	1·10	1·23	12·2	11·9
B	140	240	93	37	82	16	1,480	1,507	163	167	0·99	1·05	10·7	10·9
C	143	254	147	83	76	20	1,640	2,746	194	333	1·57	2·7	15·8	27·5

Sugar: There is an increase of reducing substances in both phases. This affords an interesting comparison with the blood of lactating cows, in which it has been shown (Widmark and Carlens, 1925) that there is a hypoglycemia varying as the amount of milk produced. In the cow the mammary glands apparently deplete the blood sugar in order to form lactose. In the opossum the hyperglycemia noted may be due to liver damage and impaired glycogenic function (it is noteworthy that animal C, with its damaged liver, shows the greatest hyperglycaemia), or it may be due to increased sugar being poured into the blood to meet the demand for sugar to be secreted into the milk. The nature of the sugar present was not determined, but since the opossum normally shows a pentosuria, some of the circulating substance is presumably pentose.

Non-protein Nitrogen: The N.P.N. remains unchanged in animals A and B: apparently the nitrogen supply is adequate for the output in the milk; the high N.P.N. of animal C is probably attributable to its hepatic condition.

Total P.: This is unaltered by lactation.

TABLE 2B.

Lipoid Constituents of the Glands of Lactating Opossums.

Gland.	Animal.	Dry weight. gm.	Total Lipoids. gm./100 gm.	Cholesterol. mg./100 gm.	Fatty Acids. mmoles/100 gm.	Lipoid P. mg./100 gm.
Kidney	A	—	15·1	913	25·7	270
	B	—	14·7	1,064	23·2	242
	C	—	19·2	1,257	24·2	232
Spleen	A	—	13·5	1,543	16·4	243
	B	—	14·7	1,396	17·8	260
	C	—	15·4	1,693	22·4	220
Liver	A	—	15·6	730	25·8	223
	B	—	16·0	896	24·9	230
	C	—	31·6	1,148	71·0	315
Adrenals	A	L 0·0709	41·6	2,818	17·7	—
		R 0·0528	37·9	3,908	12·7	—
	B	0·0783	42·0	4,052	12·4	—
	C	0·0677	43·2	5,273	11·5	—

Lipoids: In cows it has been shown that the amount of lipoids in the blood corresponds to the amount produced in the milk. In rabbits suckling their young, cholesterol rises and persists during lactation (Coppola, 1935). In humans, blood fatty acids rise during pregnancy (Elmindorff and Effkemann, 1935, and Bockelmann and Bock, 1934) and show a sharp fall after delivery (Elmindorff and Effkemann). The healthy opossums A and B show no blood or gland lipoid changes consequent on lactation. Specimen C, with her degenerate fatty liver, shows a striking increase of plasma over cell lipoids, which applies to all three fractions; the total lipoids and cholesterol of the kidney, spleen and liver are increased, the fatty acid content of the spleen is somewhat raised and the fatty acid content of the liver is increased almost three times. The adrenals show a somewhat increased cholesterol content. Similar lipoid changes have been demonstrated by workers in the dietary production of fatty livers, and it would appear that animal C is exhibiting an altered chemistry atypical of lactation and attributable to the condition of its liver, or to the factors from which its liver condition arises.

Adrenals: The increase in the dry weight of the adrenals is interesting. Specimen A, whose young was embryonic, had adrenals more than twice the average weight, while the other two animals in the later stages of lactation still exhibited enlarged adrenals. An increase in weight of the adrenals during pregnancy has been observed by many workers on the mouse, rabbit and guinea-pig (Grollman, 1936). In dogs the suprarenals enlarge during pregnancy and di-

œstrus (Barnes, 1935). Grollman inclines to the view that the increased weight results from the increased lipoid store due to the increase of blood lipoids in pregnancy: it is accepted that the adrenals generally reflect the lipoid content of the blood. On the other hand, Coppola (1935) observed changes in the weight of the suprarenals of pregnant, parturient and lactating rabbits, varying inversely with the blood cholesterol. In lactating opossums A and B, with enlarged adrenals, the blood lipoids are unaffected. Although the adrenal cortex is credited with playing a part in carbohydrate metabolism, it seems rather far-fetched to associate their enlargement here with the hyperglycemia noted above. It is more probable that the increase of size in this case is referable to the androgenic tissue. Bourne (1934) describes a relative hypertrophy of the "beta" zone in the adrenals of lactating and pregnant opossums.

SUMMARY.

1. Some normal values for sugar, total phosphorus and non protein nitrogen in the blood, and for blood and gland lipoids, have been determined in the Australian opossum. The findings are compared to those in other mammals.

2. The same analyses have been carried out on the lactating animal; contrary to the findings in other mammals, no lipoid changes were observed while there was an increase in blood sugar.

3. The dry weight of the adrenals is increased during lactation.

REFERENCES.

Barnes, J. F. (1935): Anat. Rec., No. 4 & Suppl., p. 3.
Bloor, W. R. (1916): J. biol. Chem., 25, p. 577.
Bodansky, A. (1932): J. biol. Chem., 99, p. 197.
Bockelmann, O. and Bock, A. (1934): Arch. Gynakol., 158, p. 505.
Bourne, G. (1934): Nature, 134, p. 664.
Boyd, E. M. (1933): J. biol. Chem., 101, p. 323.
Britton, S. W. and Silvette, H. (1935): Science, 82, p. 230.
Coppola, M. (1935): Arch. farmacol. sper., 49, p. 232.
Elmindorff, H. R. S. and Effkemann, G. (1935): Arch. Gynakol., 160, p. 317.
Grollman, A. (1936): Monograph on the Adrenals, p. 226.
Kuttner, F. and Lichenstein, L. (1932): J. biol. Chem., 95, p. 661.
Mitchell, M. L. (1931): Austral. J. exp. Biol., 8, p. 237.
Neumann, A. (1902): Z. physiol. Chem., 37, p. 115.
Okey, R., Gillum, H. and Yokela, E. (1934): J. biol. Chem., 107, p. 207.
Page, I. H., Kirk, E., Lewis, W. H., jnr., Thompson, W. R. and Van Slyke, D. D. (1935): J. biol. Chem., 111, p. 613.
Parhon, C. I., Ornstein, J. and Sibi, M. (1933): Bull. Soc. Roum. Neurol., 14, p. 190.
Peters, J. P. and Van Slyke, D. D. (1933): Quantitative Clinical Chemistry, Vol. 2, p. 462.
Shope, R. E. (1928): J. biol. Chem., 78, p. 107.
Widmark, E. M. P. and Carlens, O. (1925): Biochem. Z., 158, p. 3.

STUDIES ON THE OPOSSUM *(TRICHOSURUS VULPECULA)*

II. THE EFFECTS OF SPLENECTOMY, ADRENALECTOMY AND INJECTIONS OF CORTICAL HORMONE.

by

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Although a good deal of experimental work has been done, much confusion exists at present with regard to the functions of the spleen and of the adrenal cortex. There are many contradictory reports as to the effects of adrenalectomy on the lipoids of the organism, while the situation concerning carbohydrate and electrolyte control by the adrenal cortex is still controversial. As far as the spleen is concerned there are a few reports connecting it with the chemistry of the blood, but nothing to establish any definite function in this aspect. Various workers have noted different chemical changes consequent on splenectomy, and it has been assigned both active and passive rôles in lipoid metabolism. Some workers have observed a reciprocity between the spleen and the adrenals, more particularly with regard to the manufacture and deposition of lipoids.

The work reported here is an attempt to discover something of the functions of the spleen and of the adrenal cortex in the Australian opossum. Lipoid analyses of the blood and glands, and sugar, non-protein nitrogen, and total phosphorus estimations of the blood, have been carried out in order to ascertain if these values are affected by such experimental procedures as adrenalectomy, administration of cortical hormone, and splenectomy, and to compare any effects obtained with those found in other mammalian species.

The general experimental procedures followed have been given, and the normal findings indicated, in the preceding paper.

THE EFFECTS OF SPLENECTOMY.

Four large male opossums were splenectomized under ether. They stood the operation well, took their food on the following day, and were returned to the colony after ten days, where they maintained good health. A month to two months after splenectomy they were sacrificed by drowning, and their blood and tissues examined.

TABLE 1A.

Blood Constituents of the Opossum after Splenectomy.

Sugar. mg./100 gm.		N.P.N. mg./100 gm.		Total P. mg./100 gm.		Total Lipoids. mg./100 gm.		Cholesterol. mg./100 gm.		Fatty Acids. mmoles/ 100 gm.		Lipoid P. mg./100 gm.	
Cells	Plasma	Cells	Plasma	Cells	Plasma	Cells	Plasma	Cells	Plasma	Cells	Plasma	Cells	Plasma
76	126	109	54	122	22	1,000	1,400	109	148	0·84	1·20	10·52	10·58
64	118	147	83	70	20	1,370	1,830	120	130	0·65	0·73	9·87	10·32
70	120	93	37	84	19	980	1,004	94	106	0·72	0·89	10·8	11·0
82	130	120	59	98	23	1,200	1,230	98	112	0·89	1·00	9·8	9·8

Summing up the results in the above table, and comparing them with the previously reported normal findings, it is seen that the amount of reducing substances and of total phosphorus in the blood is unaltered a month to two months after splenectomy; the non-protein nitrogen content is approximately doubled: and the lipoids are decreased—the total lipoids, the fatty acids and the cholesterol being lowered, on averaged values, about 40 p.c., and the lipoid phosphorus about 20 p.c.

TABLE 1B.

Lipoid Constituents of the Glands of Splenectomized Opossums.

Gland.	Dry Weight.	Total Lipoids. gm./100 gm.	Cholesterol. mg./100 gm.	Fatty Acids. mmoles/100 gm.	Lipoid P. mg./100 gm.
Liver	—	16·98	466	26·2	154
	—	13·90	954	27·8	267
	—	12·8	607	24·3	200
	—	15·4	508	20·8	190
Kidney	—	14·8	1,132	24·3	233
	—	13·1	1,281	27·01	253
	—	15·8	1,340	19·9	270
	—	12·4	1,080	24·8	225
Adrenals	0·0457 gm.	46·4	3,697	138	—
	0·0520 gm.	38·0	6,566	100	—
	0·0518 gm.	41·4	4,028	147	—
	0·0483 gm.	40·8	3,900	129	—

From the above it is seen that, in the liver, the total lipoids are slightly lowered (about 12 p.c.), the fatty acids are unaffected, and the cholesterol and the lipoid phosphorus are lowered about 25 p.c. The lipoids of the kidneys and adrenals appear unchanged.

One would anticipate that splenectomy, by causing an anaemia and reduction in blood volume, would lead to an alteration in the concentration of the blood constituents. Unfortunately, in these experiments the blood counts were not followed, so it is not known what, if any, degree of anaemia developed.

Blood Sugar: An alteration of the blood sugar consequent on splenectomy would be expected from the work of Rubegni (1935), who found that injections of

spleen extract produced hypoglucemia while splenectomy caused a hyperglucemia, and of others, who have noticed that the blood sugar in various animals is raised for periods up to one month after splenectomy. In the opossum, as we have seen, the blood sugar is unaltered a month after splenectomy. Kerly and Reid (1935), finding that the glycogen-forming power of the liver was diminished after splenectomy, attributed this to the anaemia consequent on removal of the spleen; and it seems probable that the transient hyperglucemias and other observed effects of splenectomy on carbohydrate metabolism merit the same explanation.

Non-protein Nitrogen: Sandberg and Perla (1934) noted that nitrogen retention was increased in rats after splenectomy, and Shigeshi Nagasawa (1935) found an increase of blood NH_3 in splenectomized rabbits. In the opossum, the blood non-protein nitrogen is found to be markedly increased a month after splenectomy.

Apart from a possible concentration effect due to reduction of blood volume, other procedures (e.g. adrenalectomy) raise the blood N.P.N., and it may be that this is a general effect on the animal's health rather than a special effect of splenectomy; although Yoshikatsu Yamamoto (1936) finds alterations in the distribution of the nitrogen constituents of the blood after injections of spleen extract and after splenectomy, which he interprets as connecting the spleen. through the liver, with nitrogen metabolism.

Lipoids: That the spleen has some connection with lipoid distribution seems certain. In Niemann's and in Gaucher's diseases, the lipoids of the spleen are grossly increased. Normally, there is a direct proportion between the number of lipoid-containing cells of the spleen and the lipoid content of the serum, and the endothelial cells of the spleen seem to deal directly with circulating and locally-formed fats (see Piney, 1931). On the experimental side the evidence is conflicting. Shirato (1934) found an increase in the total blood fatty acids and cholesterol, and Ligas (1935) noted raised blood cholesterol in the rabbit after splenectomy; while Yoshio Soneda (1934) found that splenectomy caused a fall in the fatty acid, cholesterol and lecithin content of the glands, lungs and muscle. Ligas considers that there is a correlation between the spleen and the adrenals affecting the blood cholesterol, and that the spleen is merely a depot for, and does not synthesize, cholesterol. On the other hand, Minovici and his collaborators (as summarized by Brose, 1936) present good evidence to show that the spleen is capable of synthesizing cholesterol; that being so we would expect to find, as noted above in the opossum, that removal of the spleen diminished the cholesterol of the blood and of depots such as the liver.

No correlation with the adrenals can be deduced from the above results. Both Ligas and Shinsaku Tsujioka (1935) noted that the adrenals in the dog enlarge after splenectomy. In the opossum the average dry weight of both adrenals after splenectomy was 0·0494 gm., as against 0·0453 gm., the average dry weight of the controls—which is not a sufficiently definite increase to suggest an enlargement of the adrenals to compensate for the removal of the postulated co-related lipoid factory, the spleen.

THE EFFECTS OF ADRENALECTOMY.

For adrenalectomy, a group of five adult males was used. Since a group of animals was not available for experimenting with other anaesthetics, ether was used. The opossum had been found to stand ether anaesthesia and surgical procedures remarkably well. As ether is contra-indicated for adrenalectomy (Grollman, 1936b), it seems probable that the short survival time after total adrenalectomy was due in part to the use of this anaesthetic.

The operation was performed in two stages; the large left adrenals were removed through a lumbo-dorsal incision and after 10 days in hospital cages the animals were returned to the colony for 1–2 months. They maintained their normal health, the smaller right adrenals being apparently sufficient for their needs.

Two of the animals were sacrificed one month after unilateral adrenalectomy. It was found that the right adrenals were slightly heavier and had a somewhat higher total lipoid and cholesterol content than the right adrenals in the normal range. The blood constituents, and the lipoids of the spleen and kidneys, were unchanged. In both cases the liver showed an increased cholesterol content.

The second adrenal was removed from the other three animals in the second month. The animals were alert and well a few hours after the operation. After 6 to 10 hours, however, they became apathetic and showed progressive asthenia. Intra-muscular injections of normal saline showed no beneficial effect. This would be expected from the findings of Britton and Silvette (1935), who demonstrated that serum sodium and chloride values are increased in the American opossum instead of decreased, as in other species, after adrenalectomy. All the animals died between 26 to 40 hours after the operation. This survival time is even shorter than that found by Britton (1931). Apparently these marsupials are very sensitive to total adrenalectomy.

Blood specimens, unfortunately, were not obtained. The glands were taken for examination within half an hour of death.

TABLE 2.

Lipoid Constituents of the Glands of Adrenalectomized Opossums.

Gland.	Total Lipoids. gm./100 gm.	Cholesterol. mg./100 gm.	Fatty Acids. mmoles/100 gm.	Lipoid P. mg./100 gm.
Spleen	14·6	1,437	29·3	276
	13·2	1,188	17·4	258
	12·1	1,074	15·4	253
Kidneys	14·5	1,132	25·0	270
	14·5	1,035	20·9	290
	17·9	772	33·8	287
Liver	22·7	1,059	37	296
	16·4	1,154	27	329
	18·4	1,043	24	300

The only positive finding is the raised cholesterol content of the liver.

The connection of the adrenals with cholesterol metabolism is very confused. Earlier workers such as Baumann and Holly (1923) and Randles and Knudson (1928) found no change in blood cholesterol after adrenalectomy; Joelson and Shorr (1924) found that adrenalectomy raised the blood cholesterol. But Blanchard (1935) finds that in cats, the blood cholesterol may be raised, lowered, or unchanged but that there is an alteration in cell permeability, causing an increase in cell over plasma cholesterol. Since no blood analyses are available here, we are unable to extend the evidence with regard to the effect adrenalectomy has on the blood cholesterol—but we would expect to find that the other glands concerned with the lipoids would be affected, if the adrenals play any active part in lipoid metabolism. That such effects were not observed above in the case of the spleen may, of course, be due to the fact that the time between adrenalectomy and death was too short for any pronounced gland changes to take place; yet the liver, both after hemi- and total adrenalectomy, shows a raised cholesterol content.

If the adrenals manufacture cholesterol we would expect to find that their removal would lower the cholesterol in the blood and in gland depots. If they are themselves simply a depot—and it is difficult to see how a pair of very small glands, containing, in the opossum, a total of about 1·8 mg. of cholesterol, would constitute a very extensive store—their removal should increase the amount in other glands.

But if the adrenals concentrate lipoids for use or transformation in their own specialized activities, then we might find that the removal of this concentration and utilization base would allow the lipoids to bank up in other organs, such as the liver. In any case, if removal of the adrenals causes an increase in the cholesterol of the liver, a certain amount of reciprocity, either active or passive, between these glands is indicated. Further aspects of the adrenals and of cortical hormone in lipoid metabolism will be discussed in the next section.

THE EFFECTS OF CORTICAL HORMONE IN NORMAL ANIMALS.

Grollman (1936c) states that no physiological effects of the cortical hormone when injected into normal animals have yet been demonstrated, and warns workers that effects so obtained should be attributed to impurities in the preparation used and not to the cortical hormone itself. He considers that when the hormone is introduced in excess to the organism, it is probably rapidly secreted or destroyed. This objection, of course, probably applies to any hormone and is recognized in most hormonal investigations and therapy by giving repeated fractions instead of one entire dose. It is a matter of general experience that one can not expect definite and conclusive results from administering hormones to an organism with an adequate supply of its own.

However, since Britton and Silvette (1935) had been able to demonstrate type distinctions of salt and carbohydrate metabolism in the American opossum with

regard to cortico-adrenal function, it seemed worth while to try the effects of cortical hormone on the *Trichosurus vulpecula*. The preparation used was a proprietary one—Eschatin (Parke Davis), described as having less than one part per 200,000 of adrenalin, and containing 10 dog units per c.c., standardized by the method of Harrop *et al.*

EXPERIMENTAL.

Two adult males and two adult females, weighing about 2 kg. each, in good health, were fasted and given subcutaneous injections of 1 c.c. of Eschatin each at one hourly intervals for 3 hours; thus each animal received the equivalent of 120 grams of fresh adrenal cortex, or about 30 dog units. This is a very much smaller dose than that employed by Britton and Silvette (1932), who gave rats 4–5 c.c. per 100 gm. weight hourly for 6 hours: in later experiments they found smaller doses to be effective. An hour after the last injection the opossums were sacrificed, and the blood and tissues examined.

TABLE 3.

Blood Constituents of Eschatinized Opossums.

Animal	Sugar. mg./100 c.c.		N.P.N. mg./100 c.c.		Total P. mg./100 c.c.		Total Lipoids. mg./100 c.c.		Cholesterol. mg./100 c.c.		Fatty Acids. mmoles/ 100 c.c.		Lipoid P. mg./100 c.c.	
	Cells	Plasma	Cells	Plasma	Cells	Plasma	Cells	Plasma	Cells	Plasma	Cells	Plasma	Cells	Plasma
Males	275	320	151	33	86	22	1,580	1,833	156	146	1·05	1·63	12·5	12·8
	284	330	140	22	75	20	1,490	1,630	160	162	1·10	1·54	11·9	12·3
Females	193	224	174	58	95	23	1,466	2,266	146	154	1·05	1·64	16·0	17·0
	290	340	160	52	90	19	1,560	1,790	149	148	0·90	1·44	15·0	15·5

Sugar: Thaddea (1935) and Britton and Silvette (1932) using intraperitoneal injections of cortical hormone, were able to raise the blood sugar of normal animals: Grollman and Firor (1935), who administered the hormone orally, deny that there is any consequent rise of the blood sugar. Certain differences might arise from the route employed—the hormone may be more susceptible to destruction orally than parenterally.

It is well known that loss of carbohydrate from blood, liver and muscle follows adrenalectomy, and that replacement therapy with cortical hormone will restore the blood from hypoglycemic even up to hyperglycemic levels; and Britton and Silvette found that muscle and liver glycogen were rapidly increased by administering cortical hormone to normal rats and rabbits. The exact connection of the adrenal cortex with carbohydrate metabolism is still unknown: it is probable that it aids in absorption of carbohydrates by phosphorylation, and it appears to be connected with glycogen synthesis and lysis. In the Australian opossum the concentration of reducing substances in both phases of the blood is markedly increased by Eschatin.

Non-protein Nitrogen: The non-protein nitrogen values are high, and the distribution between cells and plasma is notably affected—whereas in the normal

animals the concentration in the cells is about twice that of the plasma, here the cells contain from three to six times as much as the plasma.

In adrenal insufficiency, it has been amply demonstrated that there is a loss of water from the blood and the tissues, which results in a decrease of the relative water content of the blood plasma. If a water shift in the reverse direction, from cells to plasma, concentrating the non-diffusible substances in the cells had occurred here, we would expect to find that glucose, being freely diffusible, would follow the water distribution and show a diminished concentration, relative to the plasma, per unit volume of cells. Actually the reducing substances have increased about 20 p.c. in the cells relative to the plasma. Grollman (chapter XI) explains the water and electrolyte disturbances in adrenal insufficiency as being due to impaired kidney function consequent on the injury to the renal tubules; if alterations in water distribution occurred under the influence of excess cortical hormone, it would be difficult to attribute it to disposal of electrolytes by the kidneys. Here it would seem more probable that an alteration of the permeability of the cell membranes themselves, concentrating non-diffusible nitrogenous and reducing substances in the cells has occurred and that, as indicated by Blanchard, the adrenals have some control of cell and membrane permeability.

Lipoids: The total lipoids and the cholesterol of the blood were unchanged after Eschatin. The plasma lipoid P averaged 14·4 mg. in the four animals of this group, as against an average of 12·1 mg. in the eight animals of the control group: the difference is within the normal range. The total plasma fatty acids average 1·56 mmoles as against the control average of 1·4—again a slight increase within the normal range.

In comparing the liver lipoids of a group of rats with those of a similar group after a single dose of 1 c.c. Eschatin, it was found that the livers of the Eschatin group had higher total lipoids, higher phospholipoids and higher fatty acids, than their controls. The cholesterol values were unchanged. In the group of opossums the liver total lipoids were raised to an average value of 23 gm./100 gm. (normal average 16·7 gm./100 gm.): the cholesterol was lowered to an average value of 452 mg./100 gm. (normal average 849 mg./100 gm.): the average fatty acid content was 38·7 mmoles/100 gm. (normal average 22·2 mmoles/100 gm.): and the lipoid P was also increased (average 334 mg./100 gm.: normal average 275 mg./100 gm.). The same effects were found in the spleen: average total lipoids 18·4 mg./100 gm.: normal average 13·4 mg./100 gm.); average cholesterol 970 mg./100 gm.: normal average 1,389 mg./100 gm.; average fatty acids 29·9 mmoles/100 gm: normal average 18·3 mmoles/100 gm.; average lipoid P, 347 mg./100 gm.: normal average 251 mg./100 gm. The lipoids of the kidneys and adrenals showed no change. In the liver and spleen, then (and perhaps in the plasma), an increase of phospholipoids has taken place; the increase in total fatty acids may be referable to an increase in phosphatide fatty acids. If the suprarenals have a "humoral mechanism" by which (presumably through the cortical hormone) they affect fat

metabolism by phosphorylation [Verzar and Laszt (1935)], there may be some connection here. Lang and Stuber (1936) found, in rabbits, that extracts from the adrenal cortex raise the blood phosphatides and cholesterol: they consider that, since injections of fatty acid esters provoke a similar effect, there is no need to assign a specific rôle to the cortical hormone in this connection.

As discussed in the previous section, conflicting evidence as to the effect of adrenalectomy on the lipoid distribution has been obtained. Similar confusion exists as to the effect of cortical hormone. Maranon and Collaza (1935) consider that blood cholesterol is a function of the activity of the adrenal cortex and find that cortical hormone increases the blood cholesterol in man; Medvei (1935) finds that the blood cholesterol in normal men is depressed by the cortical extract of Swingle and Pffifner. Reiss and Herzog (1932) found that extracts from the inter renal bodies of selachians lowered the blood cholesterol of rabbits 55 p.c. Using different fractions of cortical extract, Fiandacca and Capizzi (1934) found that one fraction was capable of raising the blood cholesterol while another lowered it.

In the opossum, the cholesterol of such factory depots as the liver and spleen is lowered after injections of cortical hormone. In the previous section it was noted that adrenalectomy raised the liver cholesterol; so we must look for some explanation of the action of the adrenals in cholesterol metabolism other than a secondary effect arising from liver damage.

SUMMARY.

1. In the *Trichosurus vulpecula*, the blood lipoids are lowered by splenectomy: they are unaffected by injections of cortical hormone.

2. The cholesterol and lipoid phosphorus of the liver are lowered by splenectomy. Adrenalectomy raises the liver cholesterol; cortical hormone lowers cholesterol in the liver and raises the lipoid phosphorus and fatty acid content of liver and spleen.

3. The reducing substances in the blood are increased after injections of cortical hormone. They are unaffected after splenectomy.

4. The non-protein nitrogen of the blood is raised after splenectomy. After administration of cortical hormone it is also raised, and the distribution between cells and plasma altered.

These findings are compared with the findings in other mammalian species, and their significance discussed.

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REFERENCES.

Amerigo Ligas (1935): Arch. farmacol. sper., 49, p. 210.
Baumann, E. J. and Holly, O. M. (1923): J. biol Chem., 55, p. 457.
Blanchard, E. W. (1935): Anat. Rec., 64, Suppl. No. 1, p. 50.
Britton, S. W. (1931): Amer. J. Physiol., 99, p. 4.
Britton, S. W. and Silvette, H. (1932): Amer. J. Physiol., 100, p. 693.
Britton, S. W. and Silvette, H. (1935): Science, 82, p. 230.
Brose, H. L. (1936): J. Cancer Research Committee, Sydney, Vol. VII, May, p. 134.
Fiandaeca, S. and Capizzi, I. (1934): Endokrinol, 14, p. 316.
Grollman, A. (1936): Monograph on the Adrenals: (a) p. 266; (b) p. 144; (c) p. 180; (d) p. 367.
Grollman, A. and Firor, W. V. (1935): Bull. Johns Hopkins Hosp., 57, p. 28.
Joelson, J. J. and Shorr, E. (1924): Arch. Int. Med., 34, p. 841.
Kerly, M. and Reid, C. (1935): J. Physiol., 84, p. 301.
Lang, K. and Stuber, B. (1936): Biochem. Z., 284, p. 270.
Maranon, G. and Collazo, S. A. (1935): Rev. Franc. Endocrinol., 13, p. 1.
Medvei, C. V. (1935): Z. klin. Med., 128, p. 58.
Piney, A. (1931): Recent Advances in Haematology, p. 10.
Randles, F. S. and Knudson, A. (1928): J. biol. Chem., 76, p. 89.
Reiss, M. and Herzog, P. (1932): Endokrinol., 10, p. 401.
Rubegni, R. (1935): Arch. fisiol., 35, p. 422.
Sandberg, M. and Perla, D. (1934): J. exp. Med., 60, p. 395.
Shigeshi Nagasawa (1935): J. med. Sci., 9, p. 191.
Shinsaku Tsujioka (1935): Arb. Med. Fakultat. Okayama, 4, p. 424.
Shirato, Y. (1934): Tohoku J. exp. Med., 23, p. 588.
Thaddea, S. (1935): Z. ges. exp. Med., 95, p. 600.
Verzár, F. and Laszt, L. (1935): Biochem. Z., 276, p. 11.
Verzár, F. and Laszt, L. (1935): Biochem. Z., 278, p. 396.
Yoshikatsu Yamamoto (1936): Nagoya J. med. Sci., 10, p. 11.
Yoshio Soneda (1934): Sei-i-kwai Med. J., 53, No. 8, p. 49.

INVESTIGATIONS ON THE SPECIFICITY OF THE ACTION OF AUXINS FOR THE AVENA AND PEA TESTS

by

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INTRODUCTION.

In view of the claim made by Went that the Avena and Pea tests are absolutely specific for auxins and the accumulating literature describing positive reactions obtained from other chemical substances, the following investigations were attempted. Pure chemical substances were chosen for testing by these two methods. The substances fall into several groups, those with chemical groupings related to the auxins, enzymes of plant origin, physiologically active substances of animal origin, and various other substances exhibiting some particular activity of physiological or chemical interest.

HISTORICAL SURVEY.

Since the isolation of heteroauxin (β indolyl acetic acid) by Kögl (1934), various attempts have been made to relate the activity of heteroauxin and the auxins "a" and "b" to some sensitive grouping of the molecules. The grouping of heteroauxin in common with auxins "a" and "b" is COOH. Kögl and Kostermans (1935) investigated tryptophane, which is the parent substance of heteroauxin, and some intermediate products, and found that alteration in certain regions of the molecule affected the activity, while other regions were indifferent to alteration.

Several substances have been reported as having growth-promoting properties. Indene-3-acetic acid and coumaryl-1-acetic acid are claimed by Thimann (1935) to be analogous in action to heteroauxin, while skatol is reported to be active by Glover (1936) but inactive by Kögl. The latter (1935) describes activity in the pyruvic acid derivative of tryptophane, and Haagen-Smit and Went (1935) consider the activity of cis-cinnamic acid to be comparable with the activity of heteroauxin. The steric isomer, cinnamic acid is however inert.

A selective activity of substances on different plants is suggested by the acti-

vity of β indolyl propionic acid in causing epinasty and root formation on tomato (Hitchcock, 1935) and its inactivity in the Avena test. Again coumaryl-1-acetic acid is active in causing curvature in peas but not in Avena. Thimann (1935) relates this differential activity to difficulty of transportation of substances in various plants.

Plate 1. Curvatures of Pea Seedlings obtained with Thionaphthene-3-acetic acid. Dilutions 1 in 20,000, 1 in 30,000, 1 in 40,000 glass distilled water. Water control above.

METHODS AND APPARATUS.

The establishment of a standard technique for tests on etiolated oat coleoptiles required a dark chamber, which could be maintained at a constant temperature, and a constant high relative humidity. A suitable chamber of a size to accommodate an adequate number of experimental plants was constructed and housed in a large dark room. Facilities were provided in the way of spectroscopically pure ruby glass windows for the examination of test plants during an experiment. The dark chamber could be opened at the top by lifting off a closely-fitting lid lined with felt.

Humidity Control.

The air in the chamber was kept at a high relative humidity by allowing water to evaporate from filter papers in several places in the chamber. Specially constructed awn hygrometers were used to measure the relative humidity, and to ensure that it was maintained at a constant figure in the chamber. These hygrometers were calibrated by placing them in a desiccator over a relatively large volume of a definite concentration of sulphuric acid, at a temperature of 25°C. The percentage of relative moisture at that temperature over the sulphuric acid solution was obtained from the International Critical Tables. The hygrometers were recalibrated at intervals of one month.

Temperature Control.

The most satisfactory temperature for obtaining growth curvatures on oat coleoptiles has been found to be 25°C. This temperature was maintained in the dark chamber by a wire heated by an A.C. electric current of 220 volts, and operated by a thermoregulator. The heating wire consisted of twelve feet of a nickel-chromium alloy wound around porcelain insulators of the cotton reel type which were fixed on to an asbestos sheet placed in the bottom of the box.

The heating wire was controlled by a mercury toluene type of thermostat filled with sulphur-free toluene to prevent the formation of any sulphide of mercury, which would tend to clog the capillary. The thermostat was carefully regulated, so that the mercury in the capillary just made contact with the platinum wire terminals at 25°C. The circuit used was a modification of that described by Leach (1931).

The heating of the air in the chamber depended on convection currents, but a very uniform temperature was obtained throughout the chamber by this method of heating. The apparatus gave a temperature variation of less than 0·1°C.

A perforated iron grid was placed on rests above the heating wire to support the trays of oats. A shelf was also made half way up the chamber so that a greater number of oats could be grown.

Technique of Oat Testing.

A combination of the methods of Went (1928) and Laibach (1933) was followed in carrying out the tests. The strain of oats used was the standard "Siegeshafer" type, obtained through the courtesy of Dr. Akerman of Swalöv, Sweden.

The oats with the glumes removed were soaked for three hours in water, then set out on moist filter paper in sterile petri dishes and placed in the dark chamber to germinate. After 48 hours they were placed in glass holders and allowed to grow for a further 48 hours, at which time the coleoptiles were from 3 to 4 centimetres in length, and ready to be used in the tests.

Agar blocks were made using powdered agar, carefully washed and redried to remove any possible traces of auxin which may have been present in the original seaweed. The method of of Thimann and Dolk (1932) was followed in the further preparation of the agar blocks.

The remainder of the operations were carried out in red light in the dark room. The agar was cut into small blocks 10·7 cubic millimetres in volume, with a special brass cutter and lifted on to a clean tile. The blocks were then brushed with melted 12 p.c. sterile gelatine, and attached to the wider side of an oat coleoptile which had previously been decapitated 5 mm. from the apex. The first leaf was pulled so that it broke off at the base. In earlier experiments tests for wound curvatures were carried out.

The control plants were treated before the test plants, all the instruments used being sterilized with alcohol. The treated plants were replaced in the constant temperature chamber for 1 hour and 50 minutes. Curvature records were obtained by photographs.

Technique of Pea Testing.

Pea seedlings were grown in the dark room until they were about 6 centimetres long. The method of Went (1934) was followed in the pea tests, the median slits extending for 3 centimetres

from the decapitated apex. The prepared pea shoots were placed in petri dishes containing 10 c.c. of the solution to be tested, care being taken to ensure that the apical cut surface was in contact with the solution.

The actual cutting of the pea stems was performed in daylight, but when transferred to the solutions they were placed in the dark room.

On placing in water or in solutions containing no growth substance, the two split halves bend outwards as a result of tissue tension, and the outward curvature becomes fixed. In an aqueous solution of a growth substance, however, the free ends of the two halves show an inward fixed curvature after 6 to 12 hours.

Plate 2. Curvatures of Pea Seedlings obtained with Thionaphthene-3-acetic acid in Pea Test. Dilutions 1 in 50,000, 1 in 100,000, 1 in 150,000 glass distilled water. Water control above.

SOURCES OF CHEMICAL SUBSTANCES.

The substances chosen for testing may be grouped as follows:

1. Heteroauxin, an active growth substance.
2. Substances with chemical groupings related to heteroauxin.
3. Enzymes of plant origin.
4. Active substances of animal origin.
5. Various substances exhibiting some particular activity of physiological or chemical interest.

Solutions of these pure chemical substances in various dilutions were supplied through the courtesy of Dr. W. Davies, of the Department of Organic Chemistry of the Melbourne University. The majority of the substances were obtained from the British Drug House either directly, or through the Australian agents.

Three of the substances, heteroauxin, β indolyl propionic acid, and thionaphthene-3-acetic acid were synthesized under the direction of Dr. Davies.

1. Heteroauxin.

The first substance tested was heteroauxin (β indolyl acetic acid) in order to standardize the technique and confirm the activity of this substance at great dilutions. Difficulty was experienced in early experiments in obtaining regular curvatures.

2. Substances with Chemical Groupings Related to Heteroauxin.

The following is a diagram of the formulae of the substances related to heteroauxin and their interrelationships.

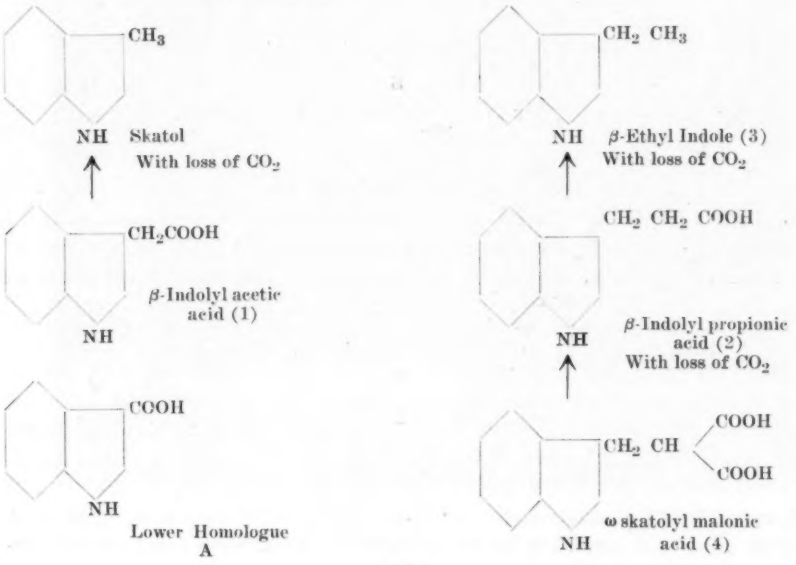


Fig. 1.

β indolyl propionic acid is the next homologue to heteroauxin, and might be expected to have similar physiological properties as the chemical properties are closely related. β indolyl propionic acid was derived directly from ω skatolyl malonic acid by removing CO_2 from the side chain in the β position. Thus it was

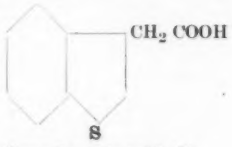
impossible that there should be any heteroauxin impurities. β indolyl propionic acid is active in producing epinasty in the petioles of treated tomato plants, and Thimann and Koepfli (1935) report a curvature on oats. Kögl (1934), however, definitely claims that the compound is inactive.

The lower homologue A has been tested by Kögl and found to be inactive on oats.

Replacement of N in Amine Group.

Thimann (1935) has reported growth-promoting properties in two substances indene-3-acetic acid and coumaryl-1-acetic acid, which contain C and O atoms respectively in place of N in β indolyl-acetic acid.

This suggestion that growth-promoting properties are not inactivated by the presence of other elements than nitrogen in the cyclic system, led to the preparation of thionaphthene-3-acetic acid.



Thionaphthene-3-acetic acid (5)

Fig. 2.

The thionaphthene group has many analogies with the indole group, and accordingly attempts have been made to prepare thionaphthene-3-acetic acid, the analogue of heteroauxin itself. A compound, M.P. 140–141° has been produced which, if not that substance itself, is isomeric with it. The final determination of the constitution of this new substance has not yet been reached.

3. Enzymes of Plant Origin.

It is considered that the curvatures obtained in the oat and pea tests are due to cell elongation which may be stimulated by alterations in the rate of metabolism brought about by enzymes. As cellulose can break down into sugars and also sugars give a cellulose-like product it was thought that the presence of an enzyme like diastase, which breaks down starch might be registered by the oat and pea tests. Similarly other enzymes affecting cell metabolism might have some growth-promoting properties.

Benzoyl carbinol, an artificial esterase, was included in this group.

4. Substances of Animal Origin.

It is possible that plants have adapted themselves to constituents of urine other than the auxins. Normal urine contains *p* hydroxyphenyl acetic acid and

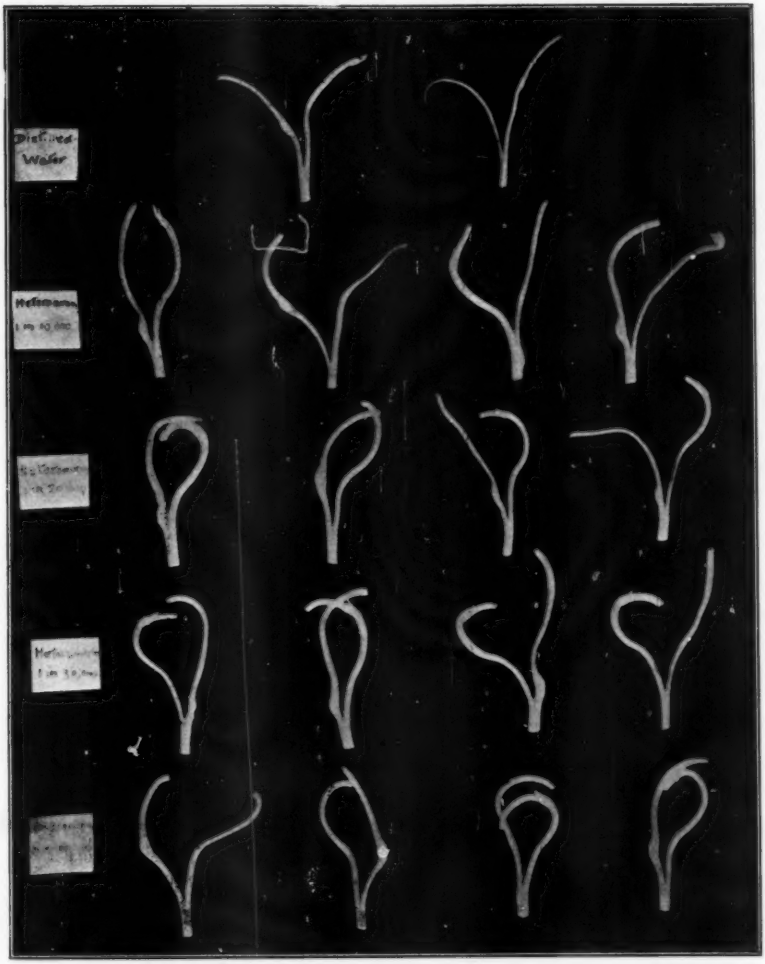


Plate 3. Curvatures of Pea Seedlings obtained with Heteroauxin 1 in 10,000, 1 in 20,000, 1 in 30,000, 1 in 50,000 glass distilled water. Water control above.

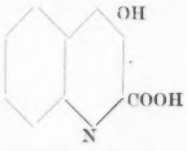
p hydroxyphenyl propionic acid, which have some similarity to auxin. Kynurenic acid, also, is constantly present in dog's urine, and has some resemblance to auxin.

There is a definite chemical similarity between the hydroxy acids and auxins, "a" and "b". Both auxin "a" and auxin "b", which are active growth hormones contain hydroxy groups and a COOH group, also the acetic and propionic groups

are biologically active so that it seemed possible that plants might be sensitive to the hydroxy acids (6) and (7) in Fig. 3 *p* hydroxyphenyl cinnamic acid has not been proved to be present in urine but is closely allied to the propionic derivative.

p hydroxy phenyl acetic acid (6)

p hydroxy cinnamic acid (7)



Kynurenic acid (8)

Fig. 3.

Apart from the above-mentioned relationships the NH group in the pyrrole ring as found in β indolyl acetic acid resembles the OH group in the phenyl ring of the hydroxy acids. This chemical similarity holds in several reactions. Thus it was expected on several accounts that different substances of animal origin might be biologically active in plant tests.

RESULTS.

Group 1. Heteroauxin.

Measurable curvatures were obtained in the oat test with solutions of synthesized heteroauxin to a dilution of one in three million in sterile glass-distilled water. This is about one-tenth of the standard value obtained by Kögl.

The results of the pea test with this compound were similar.

A range of dilutions of each substance was tested, only the greatest concentration being shown in the following tables.

TABLE 1.

Group 2. a.

Substance Tested.	Dilution.	Oat Test.	Pea Test.
ω skatolyl malonic acid	1 in 1,000	Negative	Negative
β ethyl indole	1 in 1,000	"	"
β indolyl propionic acid	1 in 1,000	"	"
Group 2. b.			
Thionaphthene-3-acetic acid	1 in 1,000 to 1 in 150,000	"	Positive

Group 3.

TABLE 2.

Substance Tested.	Dilution.	Oat Test.	Pea Test.
Zymin	1 in 1,000	Negative	Negative
Lipase	1 in 1,000	"	"
Mannose	1 in 1,000	"	"
Mannitol	1 in 1,000	"	"
Xylose	1 in 1,000	"	"
Diastase	1 in 1,000	"	"
Benzoyl carbinol	1 in 1,000	"	"
Insulin	1 in 1,000	"	"
Insulin hydrochloride	1 in 3,000	"	"
Ascorbic acid	1 in 1,000	"	"

Group 4.

TABLE 3.

Substance Tested.	Dilution.	Oat Test.	Pea Test.
Mucic acid	1 in 1,000	Negative	Negative
Pyromucic acid	1 in 1,000	"	"
p hydroxy-phenyl-acetic acid	1 in 2,000	"	"
p hydroxy-phenyl propionic acid	1 in 2,000	"	"
p hydroxy-cinnamic acid	1 in 2,000	"	"
Kynurenic acid	1 in 1,000	"	"
Uric acid	1 in 5,000	"	"
Hippuric acid	1 in 2,000	"	"
Adrenaline	1 in 1,000	"	"
Oestrin	1 in 100,000	"	"
Trihydroxyoestrin	1 in 10,000	"	"
Histamine	1 in 1,000	"	"

Group 5.

TABLE 4.

Substance Tested.	Dilution.	Oat Test.	Pea Test.
Ethanolamine	1 in 2,000	Negative	Negative
Diethanolamine	1 in 2,000	"	"
Triethanolamine	1 in 2,000	"	"
Glucosaminchlorhydrate	1 in 1,000	"	"
Prolin	1 in 1,000	"	"
Oxyprolin	1 in 1,000	"	"
Histidin monochlorhydrate	1 in 1,000	"	"
Malonic acid	1 in 1,000	"	"
Hydroxy malonic acid	1 in 1,000	"	"
Citric acid	1 in 1,000	"	"
Allyl alcohol	1 in 1,000	"	"
Ephedrin	1 in 1,000	"	"
Sperminphosphate	1 in 1,000	"	"
Mosital	1 in 1,000	"	"
Dihydracetic acid	1 in 1,000	"	"
Cinnamic acid	1 in 1,000	"	"
Cis-cinnamic acid	1 in 1,000	Positive	Positive

DISCUSSION.

As negative results were obtained with the majority of the substances tested it is quite clear that the plant responds to a restricted and definite structure for the promotion of growth. The pea and Avena tests are, however, not specific for

the auxins as claimed by Went (1934) as a positive reaction was obtained with thionaphthene-3-acetic acid, and the results of Thimann with cis-cinnamic acid were confirmed.

It would appear that the acetic acid group in the β position of the indole ring is essential for activity as a growth hormone. The molecule may be inactivated by alteration of the region. Replacement of the nitrogen in the indole ring itself either by carbon, oxygen or sulphur, however does not inactivate the growth-promoting properties of the molecule. This indicates that there may be a wide range of growth-promoting substances in which other elements than nitrogen may be in the cyclic system.

The negative results obtained with enzymes and substances of some particular physiological or chemical interest emphasize the selectivity of plant growth reactions.

REFERENCES.

Glover, J. (1936): Nature, 137, p. 320.
Haagen-Smit, A. J. and Went, F. W. (1935): Proc. Acad. Sci. Amst., 38, p. 852.
Hitchcock, A. E. (1935): Contrib. Boyce Thompson Inst., 7, p. 87.
Kögl, F. (1934): Z. phys. Chem., 228, p. 90.
Kögl, F. and Kostermans (1935): Z. phys. Chem., 235, p. 201.
Laibach, F. (1933): Ber deut Bot. Ges., 51, p. 386.
Leach (1931): Annals of Botany, 45, p. 373.
Thimann, K. V. (1935): Proc. Acad. Sci. Amst., 38, p. 896.
Thimann, K. V. and Dolk, H. E. (1932): Proc. Nat. Acad. Sci., 18, p. 30.
Thimann, K. V. and Koepfli (1935): Nature, 135, p. 101.
Went, F. W. (1928): Rec. Trav. Bot. Neerl., 25, p. 1.
Went, F. W. (1934): Proc. K. Acad. Wetensch. Amst., 37, p. 847.

THE DENATURATION OF HAEMOGLOBIN

by

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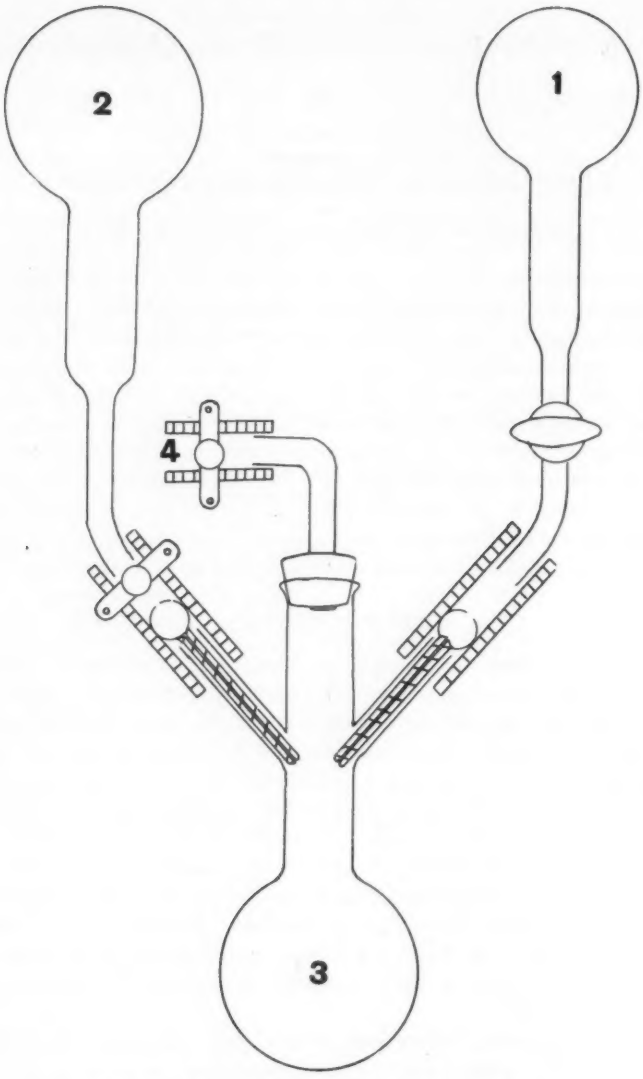
In a previous paper (Holden, 1936) it was shown that methaemoglobin, unlike oxyhaemoglobin, could be converted into a compound insoluble in water, which on solution in dilute acid or alkali could all be renatured to form methaemoglobin. In the case of oxyhaemoglobin only about two-thirds can be so renatured, the remainder having suffered another change rendering its denaturation apparently permanent. In the present paper are recorded some observations on the denaturation of haemoglobin carried out in an atmosphere from which as far as possible all traces of oxygen were excluded until after the renaturation of the renaturable protein was completed. The agents employed for the denaturation were hydrochloric acid, sodium salicylate and ethyl alcohol. The haemoglobin denatured by the first was renatured with sodium hydroxide, and by the others with water.

EXPERIMENTAL.

The apparatus used is illustrated in the accompanying figure. It consisted of three round-bottomed flasks so united that the liquids within could be equilibrated with the same gas, and the liquids in two of the flasks could be successively added to that in the third. The hydrogen used for displacing the air was stored over an alkaline solution of sodium hydrosulphite for a fortnight before use. In order to economize this hydrogen, the first two equilibrations were done against commercial hydrogen from a cylinder. The subsequent four were done using the pure hydrogen. Each equilibration lasted 15–20 minutes during which the apparatus, which was clamped to a rod, was rotated about 40 times a minute about a horizontal axis parallel to that of the reaction flask. After removal of the oxygen, the connections between the flasks were closed, and the reaction flask evacuated in order to facilitate the flow of the denaturing and renaturing agents during the subsequent operations.

Like oxyhaemoglobin haemoglobin is less easily denatured than methaemoglobin, requiring more both of acid and of sodium salicylate for complete denaturation. In order to ensure complete denaturation a little more than was found sufficient for oxyhaemoglobin was always used. Attempts were made to observe the end point of the denaturation with a direct-vision spectroscope. The spectrum of reduced acid haematin consists of a single ill-defined band not sufficiently differen-



Apparatus for the Denaturation of Haemoglobin.

1. Denaturant.
2. Renaturant.
3. Reaction flask to contain the haemoglobin.
4. Connection to pump and hydrogen supply.

tiated from that of haemoglobin for this purpose. The colour is a reddish-brown, quite different from the purple hue of haemoglobin. Sodium salicylate converted haemoglobin into denatured globin haemochromogen and the change of spectrum could be much more sharply observed. With ethyl alcohol haemochromogen was formed apparently with some reduced haematin, according to the pH of the haemoglobin solution. When sufficient acid appeared to have been added a little more was run in and the tap closed. The amounts of acid and of alkali used were determined by weighing the flasks before and after the experiment. Any experiment in which less acid had been added than was known to be requisite for the denaturation of an equivalent amount of oxyhaemoglobin was rejected. After five minutes the addition of alkali was begun. In some experiments enough was added rapidly to precipitate all the protein which was then redissolved either in acid or in alkali. In other experiments the alkali was added cautiously, till about three-quarters of the acid had been neutralized. Whether the protein had been precipitated or not the renaturation in acid was slow, extending over several hours at 20° C. The renaturation in alkaline solution was apparently immediate, though a little less quantitative.

The determination of the proportion renatured was made by ultra-violet spectrophotometry, using the Soret band as described in the previous paper. No attempt was made to separate the irrenaturable from the renatured protein. In the experiments with acid and with ethyl alcohol as denaturants the pigment was converted to methaemoglobin before photography. The tabulated results indicate nearly quantitative renaturation. In those with sodium salicylate this was not possible owing to the fact that in acid solution the protein is precipitated while in alkaline solution, the spectra of alkaline haematin and of alkaline methaemoglobin are too similar. The spectro-photometric determinations (as oxyhaemoglobin) are in consequence of less value.

In an experiment, not included in the table, in which an attempt was made to find conditions suitable for photography as methaemoglobin, the renaturation of 85 p.c. was observed but the renatured haemoglobin was undergoing steady denaturation during the determination.

In support of the view that renaturation is as complete in this case as in the others the following considerations are urged:

1. The colour of the solution was that of oxyhaemoglobin.

2. Its visible spectrum was that of oxyhaemoglobin and showed no trace of methaemoglobin.

3. On subsequent reduction of the alkaline solution no more haemochromogen was observed than when the denaturation was effected with acid (a faint trace in either case).

4. None of these criteria are fulfilled when oxyhaemoglobin is denatured by sodium salicylate.

The details of the individual experiments are set forth in Table 1.

TABLE 1.

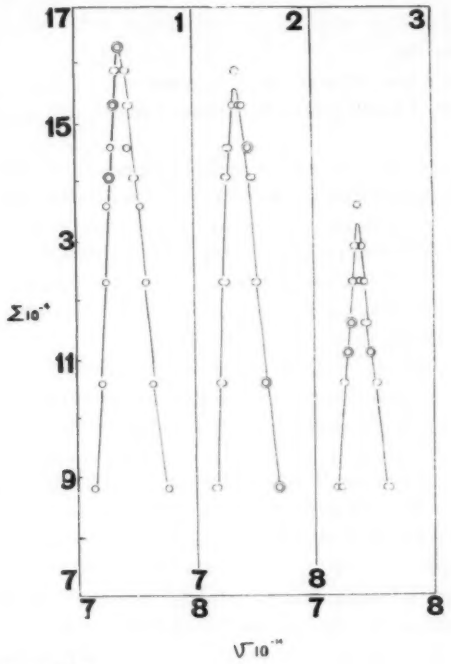
Experimental Details of the Renaturation of Denatured Haemoglobin.

Species.	Haemoglobin in grams.	Dissolved in c.c. H_2O.	Gas in flask.	Denaturant.	Volume in c.c.	Renaturant.	Volume in c.c.	Percentage Renatured.	Remarks.
Rabbit	0·14	4	H_2	0·1 N. HCl	4·5	0·1 N. NaOH	5·1	90	Renatured in alkaline solution after precipitation.
Rabbit	0·14	4	H_2	0·1 N. HCl	3·35	0·1 N. NaOH	3·83	95	Renatured in alkaline solution after precipitation.
Rabbit	0·07	2	air	0·1 N. HCl	2·0	0·1 N. NaOH	2·4	70	Renatured in alkaline solution after precipitation.
Horse	0·13	4	H_2	0·1 N. HCl	5·2	0·1 N. NaOH	5·4	90	Renatured in alkaline solution after precipitation.
Horse	0·065	2	air	0·1 N. HCl	2·0	0·1 N. NaOH	2·4	50	Renatured in alkaline solution after precipitation.
Horse	0·13	4	H_2	0·1 N. HCl	5·65	0·1 N. NaOH	5·03	90	Renatured in acid solution without precipitation.
Horse	0·065	2	air	0·1 N. HCl	2·0	0·1 N. NaOH	1·5	60	Renatured in acid solution for 5 hours without precipitation.
Ox	0·091	4	H_2	0·1 N. HCl	3·68	0·1 N. NaOH	3·05	95	Renatured in acid solution after precipitation.
Ox	0·024	1	air	0·1 N. HCl	0·8	0·1 N. NaOH	0·65	70	Renatured in acid solution without precipitation.
Ox	0·118	5	H_2	0·1 N. HCl	3·51	0·1 N. NaOH	3·01	87	Renatured in acid solution without precipitation.
Ox	0·047	2	air	0·1 N. HCl	2·0	0·1 N. NaOH	1·8	75	Renatured in acid solution without precipitation.
Ox	0·118	5	H_2	Heat to 90°C. and cool to 20°C.				40	
Ox	0·091	4	H_2	Sodium Salicylate 3·65 g.	6·37	H_2O	30·0	95	The denatured form appeared to be all haemochromogen.
Ox	0·118	5	H_2	Sodium Salicylate 3·45 g.	5·54	H_2O	30·0	95	The denatured form appeared to be all haemochromogen.
Ox	0·0277	1	air	Sodium Salicylate 0·623 g.	1·0	H_2O	10·0	70	
Ox	0·091	4	H_2	Ethyl Alcohol	10·0	H_2O	30·0	85	The denatured form appeared to be a mixture of haemochromogen and reduced haematin.
Ox	0·111	4	H_2	Ethyl Alcohol	10·0	H_2O	30·0	85	The denatured form appeared to be a mixture of haemochromogen and reduced haematin.
Ox	0·0277	1	air	Ethyl Alcohol	2·5	H_2O 0·1 N. NaOH	8·0 0·4	75	

DISCUSSION.

There is evidence that when oxyhaemoglobin is treated with acid about one-quarter of the oxygen is absorbed by some group other than the reduced haematin. At the same time about one-quarter of the protein is immediately irrenaturably denatured. The fact that methaemoglobin suffers little rapid irreversible denaturation by acid compared with oxyhaemoglobin, might mean either that the globin of the former differs in constitution slightly from that of the latter and is accord-

ingly less susceptible to oxidation, or that the rapid irreversible denaturation observed with the latter is to be attributed to an oxidation of the protein coupled with that of the reduced haematin.



Ordinates = $\Sigma 10^{-4}$
Abscissae = $\nu 10^{-14}$

$$\Sigma = \frac{M}{cd} \log \frac{I_0}{I}$$

M = equivalent weight (16,700).
c = No. of grams in one litre.
d = Depth of cell in cm.
I_0 = Intensity of entering beam.
I = Intensity of emergent beam.

1 = Control methaemoglobin.
2 = Renatured haemoglobin as methaemoglobin.
3 = Renatured oxyhaemoglobin as methaemoglobin.

The graph relates to the experiments using ox haemoglobin with hydrochloric acid as the denaturant.

The fact that haemoglobin can be denatured by acid and renatured in as high a proportion as methaemoglobin renders the latter hypothesis more acceptable. Wu and Lin's (1927) lower figure was probably due to the presence of traces of oxygen.

If the figures of Roaf and Smart (1923) be accepted the complete oxidative irrenaturable denaturation of an equivalent of globin would appear to require one molecule of oxygen. This oxidation can proceed slowly at any pH but is more rapid in alkaline than in acid solutions. If, however, both reduced haematin and oxygen are also present a coupled reaction ensues with very rapid oxidative irrenaturable denaturation.

There are doubtless other types of irrenaturable denaturation. The product of the denaturation of haemoglobin by heat contained only some 40 p.c. of renaturable protein.

The fact that so much of the work in this field has been done on oxyhaemoglobin has undoubtedly tended to make for the view that denaturation is one single reaction common to a number of types of native proteins, whereas in fact it probably covers a number of different reactions with different resultants. The only common factor (which however is probably capable of further analysis) is the presence in the protein of unstable groups of a character which is yet obscure though evidence accumulates to show that they contain sulphur.

With regard to the possibilities suggested in the paper by Wu and Lin (1927) that reduction by sodium hydrosulphite may increase the amount of protein renatured it must be remembered that oxyhaemoglobin and CO-haemoglobin have much more striking spectra in the field of the direct-vision spectroscope than have methaemoglobin or haematin. It has never been the author's experience that, when careful quantitative determinations were made by spectrophotometry of the whole protein present, the use of a reducing agent materially increased the actual yield of renatured protein.

It would appear a reasonable hypothesis that the second (and irreversible) step in the destruction of globin is an oxidation but there seems no important evidence that any chemical agent so far used has any power to reverse it.

ACKNOWLEDGMENT.

The author desires gratefully to acknowledge the loan of a spectrophotometer by Professor W. A. Osborne, Professor of Physiology in the University of Melbourne.

SUMMARY.

Haemoglobin has been denatured in an atmosphere of hydrogen by acid, ethyl alcohol, and sodium salicylate. Denatured (reduced) haemoglobin can be renatured in practically as high a proportion as methaemoglobin, thus offering a sharp contrast to the behaviour of oxyhaemoglobin.

REFERENCES.

Holden, H. F. (1936): Austral. J. exp. Biol., 14, p. 291.
Roaf, H. E. and Smart, W. A. N. (1923): Biochem. J., 17, p. 579.
Wu, H. and Lin, K–H. (1927): Chinese J. Physiol., 1, p. 219.

A COMPARATIVE STUDY OF THE INACTIVATION OF A BACTERIOPHAGE BY IMMUNE SERUM AND BY BACTERIAL POLYSACCHARIDE

by

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Levine and Frisch (1933–34) have shown that certain bacteriophages are readily inactivated by suitable bacterial extracts. The phage inhibiting agent (P.I.A.) of the extracts is a polysaccharide substance. This has been confirmed by Gough and Burnet (1934). To explain the experimental facts of inactivation of phage by both antiserum and P.I.A., Burnet (1934) has postulated a bacteriophage surface consisting of a mosaic of two kinds of active groups—A. and B.—which determine the antigenicity and virulence respectively.

The work described in this paper is an attempt to discover something of the mutual relationship of the two groups. This was done firstly, by inducing variation in one of them, and observing any effects in the other set of receptors, and secondly, by studying the combined action of antibody and P.I.A. on the phage.

A third section is concerned with the ability of P.I.A.-treated phage to be adsorbed by susceptible bacteria.

TECHNIQUE.

The phage used was Morison's type phage H., belonging to group 11 of Burnet's classification, and consisting of large particles 50–75 mμ in size. It was chosen because it was active antigenically and highly sensitive to P.I.A. The strain used for growth and titration was the original smooth Hiss and Russell strain Y. or B. dysenteriae (Flexner). In one set of experiments, the action of heat at 56°C. on the phage, titrations were also made on a rough strain of B. sanguinarium, N.C.T.C. No. 398 R.

An homologous antiphage serum was prepared by intravenous injection into rabbits of phage H. in the form of filtrate from a lysed broth culture of the strain Y.S. Four injections of increasing amounts from 1 c.c. to 2 c.c. were made at weekly intervals, and the rabbit bled eight days after the last injection. The serum was absorbed free of bacterial antibodies by incubation with a concentrated emulsion of Y.S. bacteria for one hour at 37°C., followed by overnight refrigeration. After centrifugation, the supernatant liquid was filtered through a Seitz filter and shown to contain no detectable agglutinins for Y.S.

A specimen of phage inactivating agent was prepared from Y.S. Bacteria grown for 48 hours on twelve large nutrient agar plates of 20 cm. diameter were washed off with sterile distilled

water saturated with chloroform, using 10 c.c. of water per plate. After centrifugation, the supernatant liquid was separated and the bacteria suspended in 150 c.c. of sterile distilled water. 150 gm. of urea were added, and the flask heated to 50°C. for 15 minutes at pH 7·0. The bacteria formed a gelatinous mass. The urea was dialysed away through cellophane. The nucleoprotein was precipitated with a few drops of 10 p.c. acetic acid and removed by centrifugation. The clear supernatant liquid—330 c.c.—was concentrated at 40°C. under reduced pressure to 40 c.c., neutralized and precipitated by three volumes of alcohol in the cold. The precipitate was centrifuged, washed with absolute alcohol and dried in a vacuum desiccator. The yield was 40 mg. This was not completely soluble in water. The insoluble material was removed by centrifugation, and the clear supernatant liquid used in experiments. This substance is probably impure specific bacterial polysaccharide. It gave an intense Molisch reaction and a very faint biuret test, and precipitin test to 1/100,000 dilution with Y.S. antiserum.

The effect of antibacteriophage serum and P.I.A. was tested on the phage by the usual method of mixing 0·05 c.c. of serial dilutions with an equal volume of a standard dilution of the phage. This standard dilution was made to give an easily countable number of plaques, approximately 100–250, when 0·02 c.c. of the control tube contents was plated. The mixtures were incubated at 37°C. or 45°C. for four hours, and then duplicate 0·02 c.c. lots from each tube were titrated on 1 p.c. agar plates spread with a young broth culture of Y.S. Eighty per cent. reduction of the control plaque count was taken as the end point of an experiment. Freshly grown phages were used in the experiments.

I. THE CHARACTERISTICS OF A VARIANT PHAGE INSUSCEPTIBLE TO P.I.A. INACTIVATION.

A variant of phage H. was required for observation of any changes in reactivity with P.I.A. or antiserum accompanying a modification of virulence. This variant was obtained by Schlesinger's (1932–33) technique.

The phage was grown in a concentrated emulsion of Y.S. bacteria washed from a 24 hour agar slope culture with 3 c.c. of nutrient broth at pH 8. After overnight incubation, the broth was centrifuged and a few drops of the clear supernatant liquid used to inoculate a fresh emulsion of Y.S. The re-inoculation was repeated four times.

The titre of the phage so obtained was always considerably higher than that of the original stock phage. Also the plaques were larger. Measurements were made of 50 plaques of each phage from an enlarged photograph of an agar plate, equal halves of which were spread with the two phages. The outer margins of the plaques were marked serially along the edge of a sheet of paper. The measurements could be made directly with a millimetre rule. The average diameter of a plaque of the variant H. was 1·02 mm., compared with 0·76 mm. of the stock phage.

This variant differed in most of its reactions from the typical stock phage. Its adsorption by dead homologous bacteria was very much less. A 24 hour broth culture of Y.S. was heated to 60° C. for half-an-hour, and serial dilutions of this tested against standard dilutions of phage as in P.I.A. and antiserum experiments. While its inactivation by antiserum was slightly greater than that of the stock

phage, its inactivation by P.I.A. was very much less. The data of these experiments are given in Table 1 and Fig. 1.

TABLE 1.

Comparison of the Inactivation of Phage H. and its Variant by Different Agents.

A. *Inactivation by Absorption to Heat-killed Susceptible Bacteria.*

	Final dilutions of heated broth cultures.				
Phage.	2	20	200	2,000	Titre.
Stock	0	0	14	70	350
Variant	6·5	8·5	58	82·5	50

B. *Inactivation by Anti-bacteriophage Serum.*

	Final dilutions of Serum.					
Phage.	2,000	6,000	20,000	60,000	200,000	Titre.
Stock	0	3	50	77·5	100+	9,000
Variant	0	1	20	53·5	100	20,000

C. *Inactivation by P.I.A.*

	Final dilutions of 1 p.c. P.I.A.							
Phage.	2	20	200	2,000	6,000	20,000	200,000	Titre.
Stock	3	2	3	7	21	61	88	6,000
Variant	53·5	56	93	100+	100+	100+	100+	<2

The figures represent the number of plaques to the nearest whole number, expressed as percentages of the control count, obtained from the mixtures of standard phage dilution with the reagents shown. The percentages are calculated from the average of two platings of each mixture. All experiments were carried out at 37°C. for four hours.

The variant phage is much more susceptible to heat inactivation than the original strain. Exposure to 56° C. for five minutes reduces its titre to 10 p.c. of the initial level, while similar exposure of the normal phage is practically without effect on its titre. Fig. 2 shows the course of inactivation of the two phages when highly diluted in nutrient broth at 56° C.

In this experiment the phages were titrated both on the homologous strain Y.S. and on another strain of bacteria, 398 R., on which about 75 p.c. of the number of plaques appeared. It was thought probable that heat would more rapidly inactivate phage for a heterologous strain than for the homologous one, and that in many respects the normal phage titrated on a heterologous strain might behave similarly to the variant phage titrated on the homologous strain. It will be seen that, although heating at 56° C. is apparently more effective in destroying the activity of phage H. against 398 R. than against Y.S., the difference is not nearly so marked as between the phage and its variant titrated on the same susceptible Y.S. strain.

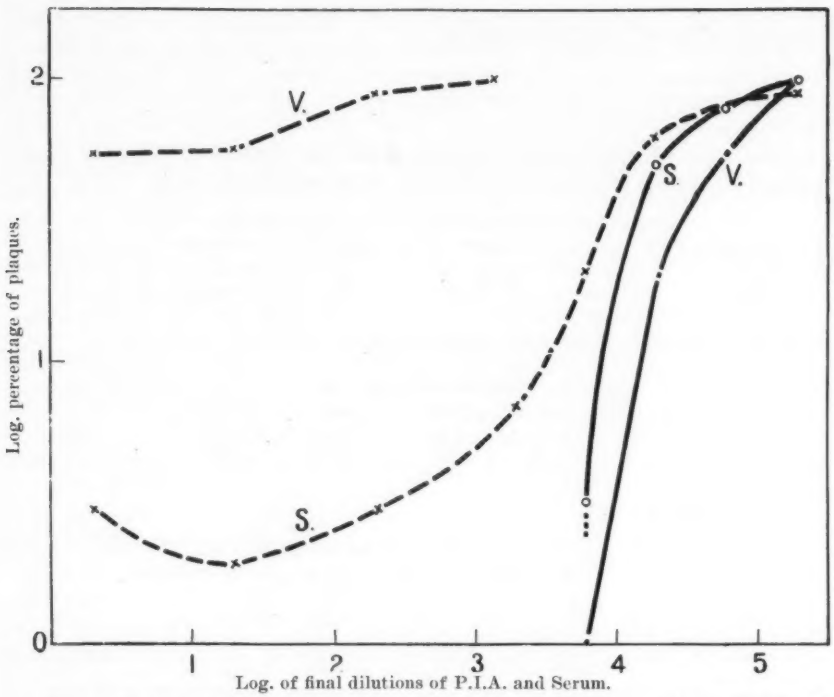

Fig. 1. To show the inactivation of phage H. and its variant by antiphage serum and by P.I.A. The logarithm of the percentage of plaques obtained is plotted against the logarithm of the final dilution of the inactivating agent. The continuous lines represent serum inactivation, and the dotted lines P.I.A. inactivation.

S. = Stock H. phage. V. = Variant H. phage.

The differences in the two phages used in the preceding experiments are summarized in Table 2.

TABLE 2.

Comparison of the Properties of Phage H. and its Variant.

Phage.	Titre.	Plaque size. Diameter.	Absorption by heat-killed Y.S. Titre.	Inactivation by antiserum Titre.	Inactivation by P.I.A. Titre.	Inactivation by heat at 56°. P.c. Survivors.		
						5 mins.	1 hour.	2 hours.
Stock H.	$4{\cdot}7 \times 10^9$ c.c.	0·76 mm.	350	9,000	6,000	92	45	13·5
Variant H.	$9{\cdot}1 \times 10^9$ c.c.	1·02 mm.	50	20,000	< 2	10·5	2	0·5

A variant phage of similar character has also been isolated from the phage C. 16. The stock phage had been grown on a rough B. coli strain and successive re-inoculations made from the supernatant fluid of centrifuged cultures without

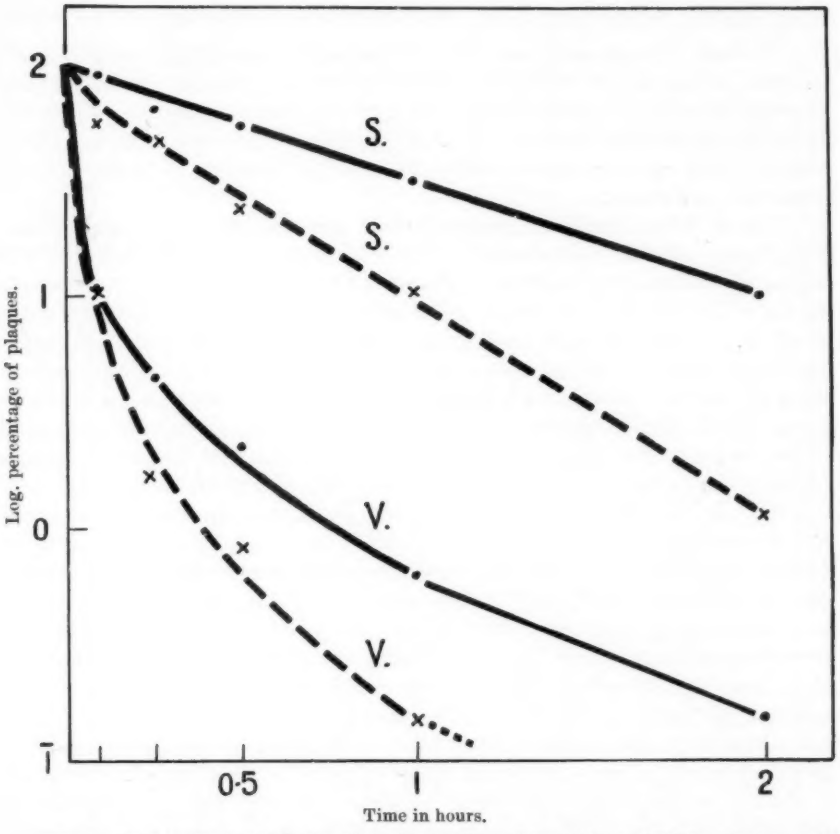

Fig. 2. To show the heat inactivation of phage H. and its variant at 56°C. The logarithm of the percentage of plaques obtained is plotted against the time in hours. The continuous lines represent the phage survivors plated on Y.S., and the dotted lines those plated on 398 R.

S. = Stock H. phage. V. = Variant H. phage.

filtration. Variant plaques appeared which had less clearly defined edges and were of about twice the diameter of the normal plaques. From one a strain of phage was isolated which was still inactivated to titre by anti-C. 16 serum, but differed from the parent strain in a similar manner to the H. variant. It was not

inactivated by a P.I.A. preparation from Y.S. which was moderately active against the original phage; neither was it absorbed by heat-killed susceptible bacteria. It was also less resistant to heat than the parent strain.

II. THE MUTUAL EFFECT OF IMMUNE SERUM AND P.I.A. ON PHAGE.

To study the mutual action of P.I.A. and anti-bacteriophage serum on stock phage H., it was subjected to preliminary treatment with these agents before titration with them in the usual manner. As early experiments suggested that serum had a definite effect on the action of P.I.A. on phage, without the converse holding, most of these experiments are concerned with the reactions of phage after its treatment with serum.

The serum used had been absorbed free of antibody to Y.S. To obviate selection effects, conditions were chosen to prevent too great an inactivation of the phage during its preliminary treatment. The use of 1/100–1/300 serum at a temperature of 1–2° C. for several hours, or at 37° C. for a few minutes, or 1/10,000 serum at 45° C. for one hour gave convenient starting material. The undiluted phage was mixed with the diluted serum and allowed to react for a definite interval. Then the mixture was diluted to a phage concentration suitable for use as a standard dilution for the ordinary titration. This dilution also stopped further action of the serum on the phage. The diluted emulsion was kept in the refrigerator overnight and titrations made with P.I.A. and serum on the following day.

In every experiment it was found that preliminary treatment with serum reduced the degree to which the phage was inactivated with P.I.A. In general, the greater the reduction in titre produced by serum, the more complete was the resistance of the "surviving" phage to inactivation by P.I.A. Such a change might have one of two explanations: either the immune serum selectively removed those particles most reactive to P.I.A., or the changed reactivity was the result of union of antibody in sub-inactivating amounts to the phage particles concerned. It will be seen from Table 3 or Fig. 3 that with phage H. the P.I.A. preparations used did not reduce the phage titre below about 1 p.c. of the original. It appears that about 99 p.c. of the particles in an average phage suspension are potentially susceptible to P.I.A., the remaining 1 p.c. being completely insusceptible. If therefore the percentage of survivors after treatment with serum was below 1 p.c., any loss of reactivity to P.I.A. might be ascribed to a selective action of serum on the susceptible 99 p.c. with survival of particles inherently resistant to P.I.A. This difficulty in interpretation was avoided by using phage, the titre of which had been reduced by serum to about half the original value.

Table 3 gives the results of an experiment fulfilling these conditions. If we consider only the most effective concentration of P.I.A. (1:20), it will be seen that the count of plaques from control phage is reduced from 100 to 1. Under the influence of serum on these 100 particles for five hours, 40 only remain capable of

producing plaques. Of these 40, 30 p.c., i.e. 12, are resistant to P.I.A., compared with the 1 of the original untreated 100 particles. It is immediately clear that the immune serum has a direct modifying effect on at least a proportion of the phage particles, and that selection of P.I.A. resistant particles has at most an insignificant effect. It may be noted here that normal rabbit serum has no effect at all on the reactivity of phage to P.I.A.

The same table shows that this preliminary treatment with serum has no influence on the subsequent behaviour to antiserum when tested in the usual way.

Preliminary treatment of phage H. with P.I.A. will reduce the reactivity of the surviving particles with P.I.A., as found previously by Burnet (1934), but has no effect on their inactivation by serum.

The results of a typical experiment are given in Table 3 and Fig. 3. The figures represent the percentage of phage surviving the P.I.A. or serum treatment.

TABLE 3.

A. *Inactivation of Serum-treated Phage with P.I.A.*

Preliminary treatment of phage.	P.c. of original phage surviving.	Final dilutions of 1 p.c. P.I.A. 2	20	200	2,000	20,000
Phage + = vol. broth 1°C. 5 hours	100	5	0·6	8	18·5	72
Phage + = vol. 1/300 Serum 1°C. 1 min.	98	3	1	8·5	24	71
" " " 2 hours	67	27	17	21	31	96
" " " 5 hours	40	35·5	30	35	43·5	95

B. *Inactivation of Serum-treated Phage with Serum.*

Preliminary treatment of phage.	P.c. of original phage surviving.	Final dilutions of Serum. 2,000	6,000	20,000	60,000	100,000
Phage + = vol. broth 1°C. 5 hours	100	0	2	20	84	92
Phage + = vol. 1/300 Serum 1°C. 2 hours	67	0	2	22	69	77
" " " 5 hours	40	0	1	17	86	95

A similar effect has been obtained with phage L. As it is not so reactive with P.I.A., the results are less striking, but there is a flattening of the P.I.A. curve after serum treatment, without any change in the serum curve.

III. EFFECT OF P.I.A. ON THE POWER OF BACTERIOPHAGE TO COAT BACTERIA.

Burnet (1933) showed that when a large amount of phage is attached to the surface of living or dead susceptible bacteria, these become agglutinable by anti-bacteriophage serum absorbed free of bacterial agglutinins. Agglutination by the corresponding antibacterial serum is usually somewhat less active than with normal bacteria.

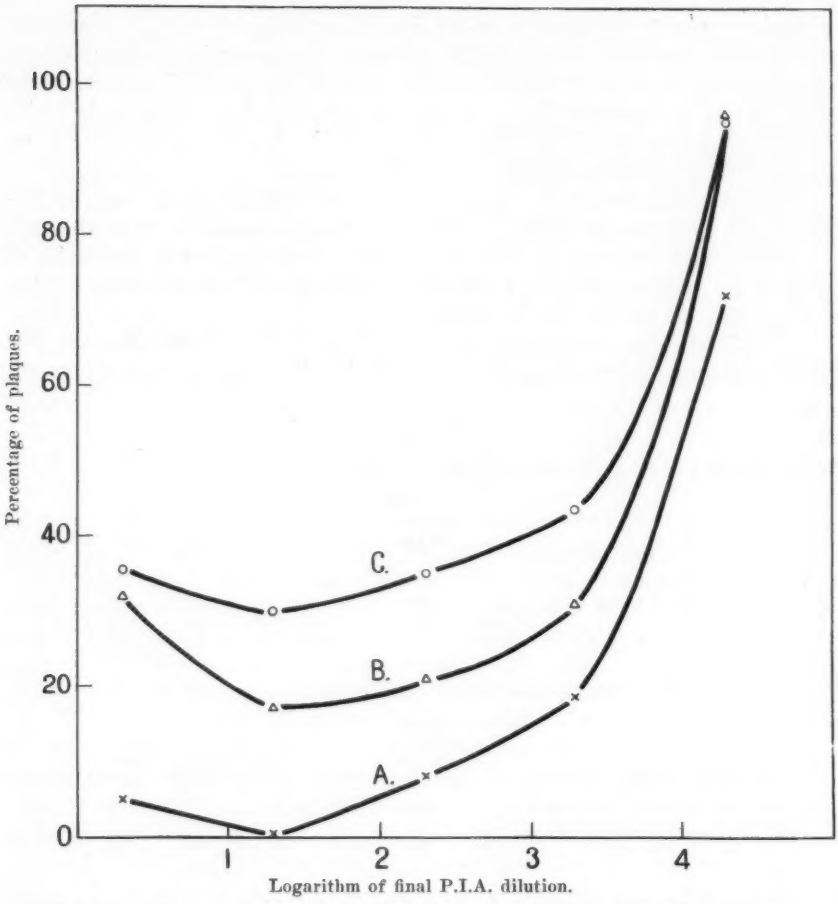

Fig. 3. To show the decreased susceptibility to inactivation by P.I.A. of phage H. previously treated with antiphage serum. The percentage of plaques obtained is plotted against the logarithm of the final dilution of P.I.A.

A. = Control phage, not treated with serum.
B. = Phage treated for two hours with 1/300 serum.
C. = Phage treated for five hours with 1/300 serum.

Phage inactivated by formalin was found still to be capable of coating bacteria in this fashion. Experiments of this sort provide the only available method of determining whether inactivated phage is or is not adsorbed by bacteria, and it was of interest to apply the technique to phage inactivated by P.I.A.

The following experiment is typical of several in which the "coating" power of phage H. was compared with that of the same phage treated with P.I.A.

A sample of phage was used which retained its original titre on keeping in the refrigerator for a week. To 9 volumes of undiluted phage, one volume of 1 p.c. P.I.A. was added and the mixture incubated for three hours at 37°C. A second similar amount of P.I.A. was added, and incubation continued for another hour, in order to make the reaction as complete as possible. Equal volumes of broth were added to the control phage. Incubation at higher temperatures had to be avoided because with many samples of phage a 30–50 p.c. reduction of the phage could occur at 45°C. The mixtures were kept overnight in the refrigerator and a titration of their plaque count made.

The bacterial suspension used was prepared from an overnight agar culture of Y.S. The bacteria were washed off in broth and centrifuged. The packed sediment was emulsified in 9 volumes of broth for use in the experiment.

Barely sufficient phage was added to make the bacteria agglutinable by H. antiserum. 0·1 c.c. of bacterial emulsion was treated with (1) 1 c.c., (2) 2 c.c., (3) 3 c.c. lots of control phage, and P.I.A. treated phage respectively. In tubes (2) and (3) each 1 c.c. of phage was added in a separate lot and allowed to react with the bacteria for 15 minutes at 37°C. before centrifugation and addition of the next lot of phage. Then the emulsions were made up to 2 c.c. in each tube with 0·2 p.c. saline containing 0·25 p.c. formalin and incubated for 30 minutes before the agglutination tests were made. These were treated at 45°C. for three hours and the final readings made after standing overnight at room temperature.

The titre of the stock phage H. used was $4{\cdot}0 \times 10^9$. A 1/20 dilution of the control phage was also used to coat the Y.S. This was of comparable titre with the P.I.A. treated phage which was $1{\cdot}7 \times 10^8$ or 4 p.c. of the control emulsion.

The agglutination results are given in Table 4.

TABLE 4.

Agglutination of Phage-coated Bacteria.

	Serum. Anti H. 1: 100. Tubes			Saline. Tubes		
Agglutinating Emulsion.	1	2	3	1	2	3
Y.S. coated with undiluted phage H.	+	+++	+++	—	±	±
Y.S. coated with phage H. 1: 20	—	—	—	—	—	—
Y.S. coated with P.I.A.-treated phage	+	+++	+++	—	+	+
Y.S.	—			—		

The results indicate that inactivation by P.I.A. does not diminish the power of phage to coat susceptible bacteria. In several experiments the bacteria coated with P.I.A.-treated phage seemed even to agglutinate more rapidly than those coated with control phage. Under these experimental conditions, phage reduced by simple dilution to a concentration slightly higher than that of the active particles in the P.I.A. mixture completely failed to coat bacteria.

Further evidence that the phage particles inactivated by P.I.A. still retain their capacity to give *in vitro* reactions with antiserum was obtained by testing their direct agglutinability with antiphage serum. The agglutination of P.I.A.-treated phage was equivalent to that obtained with control phage. No agglutination could be seen with control phage diluted 1 : 20.

DISCUSSION.

Study of the functional activity and immunological character of bacteriophages has given clear evidence of the existence of two types of specific surface structures on bacteriophage particles. Phages are active antigens, and it can be shown that the specific antibodies unite directly with the phage surface (Andrewes and Elford, 1933; Burnet, 1933). The antigenic types of bacteriophages are as diverse as those of bacteria, and the antigenic molecular groupings on their surface must be correspondingly varied. These surface groupings which determine antigenic behaviour may be termed A. groups. Another series of specifically reactive groups on the phage surface can be deduced from the capacity of phages to be adsorbed specifically by susceptible bacteria, and to be inactivated by extracts of susceptible bacteria which contain bacterial polysaccharide in a suitably active form (Levine and Frisch, 1933–34; Burnet and Gough, 1934). These may be called B. groups (directed toward the bacterium). Comparison of any considerable series of bacteriophages shows at once that the two sets of specific groupings are very largely independent. Phages lysing the same bacteria may be antigenically distinct, while antigenically similar phages may lyse quite different bacterial species.

The aim of the present study has been to seek evidence as to how the two sets of entities, A. and B., on the bacteriophage surface are related to one another. This question is of considerable theoretical importance in regard to the general problem of the way in which immune serum renders a virus or bacteriophage particle non-infective. Three groups of facts have been described above, each of which seems to contribute something to the understanding of this question. The first concerns the characteristics of a variant strain of phage developed by growth in an environment constantly containing an excess of unlysed bacteria. Under these conditions we find, in confirmation of Schlesinger (1932), that variant strains of phage appear which are less readily bound by susceptible bacteria than the normal phage. Associated, and probably correlated with this change were (1) the production of a higher final titre in broth and larger plaques on agar, (2) insusceptibility to inactivation by P.I.A., (3) somewhat increased susceptibility to inactivation by immune serum, and (4) considerably diminished resistance to inactivation by heat.

The second set of facts concerns the mutual effect of serum and P.I.A. acting on the same population of phage particles. In this sphere it has been found that phage which has been exposed to a low concentration of immune serum, but not inactivated thereby, is rendered less susceptible to the subsequent action of P.I.A. This is not a selection effect, but is a directly induced modification of the reactivity of each phage particle.

Finally, suitable experiments have shown that inactivation by P.I.A. does not prevent the phage particle concerned from becoming attached to the bacterial surface, nor modify its agglutinability by antiphage serum.

One of us has previously discussed the detailed similarities between the action of antiserum and of P.I.A. on phage in respect to such aspects as the velocity of reaction, the "percentage law", and the reduction in size of plaques produced by the "survivors" (Burnet, 1934). Any theory of the action of P.I.A. on phage must therefore be capable of accounting for these similarities. The simplest interpretation of P.I.A. effects is that union of the relatively large and heterogeneous units, molecules and fragments of bacterial surface which make up P.I.A. takes place with some of the B. receptors of the phage particle. Inactivation of phage particles to which P.I.A. is attached results from (a) the lessened chance of effective contact being made between B. receptors and bacterial surface, (b) the obstruction offered by the bound P.I.A. to the entry of the particle into the bacterium once specific union has been made, and (c) the occurrence of phage particle aggregation when a high concentration of phage is present, the larger P.I.A. units acting as links between the phage particles.

No satisfactory detailed interpretation of the phage-antiphage reaction has been published which takes account of the work of Andrewes and Elford on the neutralization reaction, and of Schlesinger (1934) and Burnet (1933) on aggregation reactions; but on the basis of this work and of recent unpublished studies, we would ascribe the inactivation of phage by antiserum to union of antibody with A. receptors of the phage surface, the observed inactivation resulting from the same three processes as have been postulated for the P.I.A. inactivation. The relative importance of the three processes probably differs considerably in the two cases, but this will be discussed later.

With this theoretical basis, the effect of small amounts of immune serum in inhibiting the action of P.I.A. may be discussed. This effect must be a result of the union of one or more antibody molecules to each of the phage particles with which we are concerned, viz. those which are *not* inactivated by serum, but rendered resistant to P.I.A. The particles modified in this fashion are still capable of making effective contact with and initiating lysis of susceptible bacteria, but have lost their original susceptibility to P.I.A. It is reasonable to deduce (1) that only a proportion of the B. receptors available for union with the living bacterial surface are capable of reacting with P.I.A., (2) that such active B. receptors are preferentially blocked by the first union of antibody to the phage particle, and (3) that this preferential blocking indicates that the most reactive A. and B. receptors are spatially contiguous on the phage particle surface, or in other words, that in some phage particles at least there are limited active areas of surface which have a greater reactivity toward both antibody and P.I.A.

This experiment also indicates that in addition to resemblances there are certain differences between the inactivations of phage by antiserum and P.I.A. respectively. Since only a proportion of B. receptors are capable of reacting with P.I.A., inactivation of phage by P.I.A. cannot result from a blocking of all B. receptors. In all probability inactivated phage can still unite normally with bac-

teria, but is prevented from proceeding to the further stages of the lytic process by the more or less mechanical hindrance of the attached P.I.A. The fact that such union to bacteria can still occur is demonstrated by the experiment in which agglutination by antiphage serum was used as an indicator of union of phage to bacteria. When corresponding experiments are made with phage inactivated by serum, the power to coat bacteria is found to be lost when the phage-antibody reaction has progressed sufficiently. This subject is still under study, but the results to date indicate that on the average slightly more antibody is needed to prevent phage particles becoming attached to susceptible bacteria than is necessary to inactivate them. In terms of the three processes involved in inactivation, either with serum or P.I.A., we would regard blocking of surface receptors and aggregation as particularly characteristic of serum inactivation, hindrance of entry into the bacterium after specific adsorption being the most important factor of P.I.A. inactivation.

The demonstration that certain areas of the phage particle surface may be more reactive toward P.I.A. than others is in accord with evidence obtained in other directions that the activity of B. receptors varies greatly within populations of the same phage, between variant stocks of one phage, and from phage type to phage type. The evidence given by Burnet (1934) in regard to the correlation between the susceptibility of a bacterial strain to phages and the range of activity of P.I.A. derived from it is sufficient to make it certain that qualitatively the same bacteriophage receptors are involved in reactions with bacteria and with P.I.A. But the rarity of phage strains which are readily inactivated by P.I.A., the complete insusceptibility of some, and the ease with which susceptibility to P.I.A. can be lost, as in the case of the variant H., can mean only that there are big differences in what may best be called the avidity of the receptors involved. Avid B. receptors, such as are present in the stock H. phage, unite readily with P.I.A.; less avid ones are uninfluenced by P.I.A., but may still initiate lysis by union with multiplying susceptible bacteria.

On this basis, our interpretation of the changes observed with the variant H. strain would be as follows: By selection in the presence of a large excess of potentially susceptible bacteria, only those phages with B. receptors of a low grade of avidity have been allowed to proliferate, and the type has become, to some extent at least, genetically fixed. Practically speaking, all the B. receptors of the variant are of the less avid type, and the observed differences between the parent strain and the variant may help to indicate something of the nature and function of the B. receptors. The variant phage multiplies at approximately the same rate as the original, but does not clear a broth culture quite so well. Despite this, the titre of filtrates is higher. This is presumably because the liberated phage particles do not so readily become attached to unlysed bacteria or bacterial fragments, and so become unfilterable. Incidentally it seems highly probable that the apparent discrepancy between the amount of phage produced per bacterium lysed in the earliest

phase of the process (Burnet, 1929) and the much smaller amount per bacterium which is found after lysis is complete may be largely due to inactivation of phage by fragments of lysed bacteria (P.I.A.) in the later stages. The increased diameter of the variant plaques on agar will similarly be ascribed to the greater mobility of phage which is not readily bound to bacterial debris.

The almost complete insusceptibility to P.I.A., and the slight absorption to heat-killed bacteria indicates both an absence of highly avid receptors and an abnormally low average avidity of all B. receptors. The greater readiness with which the variant is inactivated by heat is of particular interest, as it provides a distinct suggestion that heat inactivation is primarily due to damage of B. receptors. If heating produces progressive diminution in the avidity of the B. receptors, those phage particles would tend to survive in which the initial avidity is highest.

Increased susceptibility to inactivation by antiserum is to be expected in the variant if, as has been postulated, the function of attached antibody molecules is mainly to diminish the probability that effective contact between the B. receptors and the bacterial surface will take place. If the B. receptors are intrinsically of lower avidity, a given degree of antibody coating will be correspondingly more effective in inactivating the phage particles.

SUMMARY.

1. A variant phage strain obtained by Schlesinger's method showed, in addition to relative inability to be adsorbed to susceptible bacteria,

(i) Insusceptibility to inactivation by P.I.A.,

(ii) Increased heat susceptibility,

(iii) Slightly increased susceptibility to inactivation by antiserum, and

(iv) Gave rise to larger plaques and produced a higher titre in broth cultures.

2. Phage H. treated with subinactivating amounts of antiserum showed diminished susceptibility to inactivation by P.I.A.

3. Inactivation of phage H. by P.I.A. did not diminish its power to "coat" susceptible bacteria, and did not modify its direct agglutinability by antiphage serum.

4. These phenomena are discussed in terms of "active groups" on the bacteriophage surface.

REFERENCES.

Andrewes, C. H. and Elford, W. J. (1933): Brit. J. exp. Path., 14, pp. 367 and 376.
Burnet, F. M. (1929): *Ibid.*, 10, p. 109.
Burnet, F. M. (1933): *Ibid.*, 14, p. 93.
Burnet, F. M. (1934): J. Path. Bact., 38, p. 285.
Gough, G. A. C. and Burnet, F. M. (1934): *Ibid.*, 38, p. 301.
Levine, P. and Frisch, A. W. (1932–3): Proc. Soc. Exp. Biol. and Med., N.Y., 30, p. 993.
Levine, P. and Frisch, A. W. (1933–4): *Ibid.*, 31, p. 46.
Schlesinger, M. (1932): Z. Hyg. Infectkr., 114, pp. 136 and 149.
Schlesinger, M. (1934): *Ibid.*, 116, p. 171.

THE ACTION ON MAMMALIAN CIRCULATION OF THE SKIN SECRETION OF *HYLA AUREA*

PART II.

by

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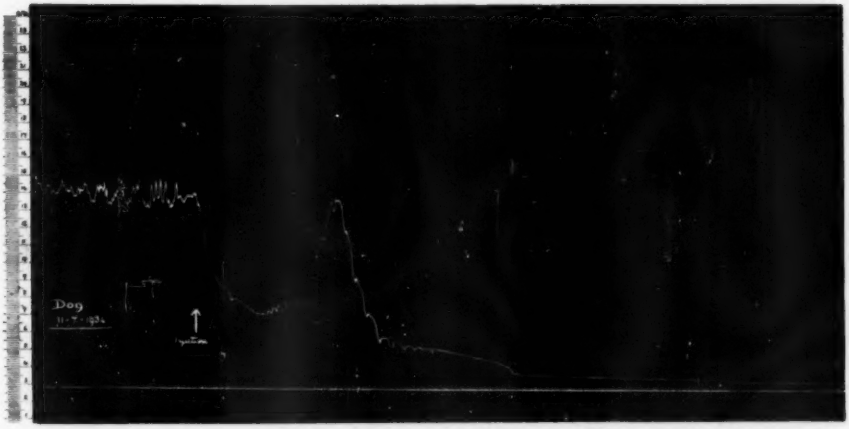
In a former communication I described the remarkable action of the secretion of the skin of *Hyla Aurea* on mammalian blood vessels (Osborne, 1930). Numerous experiments conducted since have confirmed the main conclusion then given,



Carotid pressure of dog with usual mercury manometer. Seconds time tracing indicates zero pressure. Upright arrow shows time of injection of skin secretion.

namely that the active substance when in lethal doses produces such intense constriction of the systemic arterioles that a high arterial blood pressure can persist for some time despite instant and complete cardiac collapse. I do not know of any other poisonous substance which can bring about such powerful spasm of the arterioles that escape of blood into the capillaries is prevented. Further experimentation has led me to modify my opinion that the cardiac syncope was due to tonic contraction of the myocardium. Using the heart of rabbit and cat perfused

with saline through the coronary artery by the Locke method I have been able to satisfy myself that the syncope is caused by coronary constriction.

The poison is prepared in the manner described in my first paper. A pithed frog is washed to and fro a few times in a beaker containing the usual isotonic saline. The saline is then filtered. If 100 ml. saline have been used it is found that 5 ml. of this will be lethal for the dog. On adding a little of this saline to the perfusion fluid of the isolated heart I have been able to note a sudden stop of the coronary flow notwithstanding a head of pressure of three feet. The mass of the heart remains flaccid. Should a dilute solution be employed the coronary circulation is often spontaneously restored after a few minutes' occlusion and the heart commences to beat again though it usually stops later. This explains a fact which I found rather puzzling namely that when a solution of the poison, not too strong, is injected intravenously into a dog the carotid pressure falls abruptly at first and then more gradually and that often there is then a temporary quick rise of arterial pressure due to the resuscitation of the heart. The tracing given shows this clearly. The graph represents carotid pressure with the mercurial manometer. The seconds time tracing indicates the pressure zero.

When a stronger solution is injected then the appearances are those first described, namely a complete stoppage of the heart but a very gradual fall of the blood pressure.

REFERENCE.

Osborne, W. A. (1930): Austral. J. exp. Biol., 7, p. 226.